

J Vet Diagn Invest 11:295–297 (1999)

Cross-reactivity of an automated human haptoglobin immunoturbidimetric assay for detection of haptoglobin in swine serum

Charles E. Wiedmeyer, Philip F. Solter, Charles J. Francisco, William F. Hall, Walter E. Hoffmann

The acute phase response has been previously characterized in the pig.^{2,9} Haptoglobin (Hpt), an α -2 glycoprotein, is a normal serum protein that increases 5–7 fold within 24–48 hours upon the advent of an acute inflammatory response.⁹ Serum Hpt increases as a result of natural infections and experimentally induced inflammation in pigs as in other species.^{1,2,6,9,11,12} Besides serum Hpt as a marker for inflammatory disease, it has been used as an indicator of weight gain⁴ and stress from environmental conditions.⁵ The serum Hpt concentration may therefore be useful as a monitor of overall health of pigs in a commercial production unit.

Automated assays for measuring serum Hpt have been validated for use in dogs, horses, and sheep.^{7,11,13} A commercially available immunoturbidimetric assay for human Hpt has recently been validated for use in dogs and horses.¹³ The purpose of this study is to report on the cross-reactivity of this same assay^a for use in determining serum Hpt concentrations in swine.

Serum samples were obtained from pigs housed at the Veterinary Medicine Research Farm at the University of Illinois and from samples submitted to the Purina Mills Research Center for unrelated testing. Samples from the University of Illinois were collected by venipuncture into serum clot tubes, and after centrifugation, the serum was withdrawn and stored at -70 C until assayed. Those samples submitted to Purina Mills Research Center were treated in a similar fashion. Serum used for immunoelectrophoresis, western blot, and correlation studies were from the University of Illinois, and serum for all other experiments was from the Purina Mills Research Center.

A cyanmethemoglobin (CHB) assay for serum Hpt was performed as previously described.^{3,4} Hpt was reported in milligrams of CHB binding capacity (HBC) per deciliter of serum. Samples with relatively low, medium, and high HBC were pooled and labeled respectively. The pooled samples were used for further experiments.

Immunoelectrophoresis was performed as previously described¹³ using 1- μ l aliquots of pooled swine serum containing various Hpt concentrations as determined by the CHB assay. Serum from a healthy human donor served as a control. Following standard electrophoresis on a 1.0% agarose gel film,^b anti-human Hpt antiserum (anti-Hpt antibody) was added to diffusion wells on either side of each electrophoretic lane. The antiserum was allowed to diffuse for 24 hours at room temperature, forming immunoprecipitates. The film

was washed in 0.9% saline with gentle stirring for 12 hours and stained with Commassie blue, destained in an acetic acid solution, and air dried.

For western blot analysis, serum from pigs and a human serum control were diluted 1:10 in phosphate-buffered saline and run on a 8.0% polyacrylamide gel under nonreducing conditions. Adjacent lanes containing equal aliquots of each serum sample, first incubated with excess amounts of human hemoglobin (Hb), were also run on the same gel. The gel was blotted to a polyvinylidene difluoride membrane,^c which was subsequently blocked with 5% bovine serum albumin (BSA) and incubated in anti-Hpt antibody, diluted 1:4,000 in Tris-buffered saline (pH 7.4) for 2 hours at room temperature. The membrane was then incubated with alkaline phosphatase-conjugated rabbit anti-goat IgG diluted 1:2,500 for 2 hours at room temperature. After a final wash in 1% BSA, the membrane was incubated with an alkaline phosphate substrate^d until all bands could be clearly identified.

Two models of automated chemistry analyzers^e for cross-reactivity of the immunoturbidimetric assay were used. Each machine was programmed according to the manufacturer's recommendations with the following modifications. To compensate for the ability to use undiluted serum on the older model (Hitachi 704) instrument, internal settings for the assay were altered. The alteration required a change in the mathematical model function for nonlinear curve approximations. The change is from a 4-parameter:Logit-log calculation to the 5-parameter:Logit-log. For the newer model (Hitachi 911), no internal modifications were made. For both instruments, it was necessary to dilute the lowest human standard (33 mg/dl), 1:2 (16.5 mg/dl) and 1:4 (8.25 mg/dl), to facilitate a working range of standards for ideal swine serum Hpt determination. The top 2 human standards supplied with the kit were not used. The human control was run as recommended (89–121 mg/dl) and diluted 1:2 with saline. If the older model is equipped to perform the assay, it is necessary to dilute the standard and control 1:11 with saline. Use of the newer model instrument requires no such dilution.

Pooled low- and high-HBC serum samples were run 10 times within 1 day and then once daily for 10 days to determine the within- and between-run coefficients of variation (CVs). Correlation between the immunoturbidimetric assay and the CHB assay was calculated from the results of 60 samples. Correlations between the 2 models of values obtained were made using 78 samples. Linearity of the assay was determined by diluting the pooled high serum at 10% increments with pooled low serum or saline. Dilution with pooled low serum was only performed on the newer model instrument because of depletion of sample volume. Interference from hemolysis was tested by adding increasing

From the Department of Veterinary Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, IL 61802 (Wiedmeyer, Solter, Hoffmann), Hoechst-Roussel Vet, 1407 Lincolnwood, Urbana, IL 61801 (Francisco), and Purina Mills, Veterinary Services Laboratory, 151 Danforth Drive, Gray Summit, MO 63039 (Hall).

Received for publication March 6, 1998.

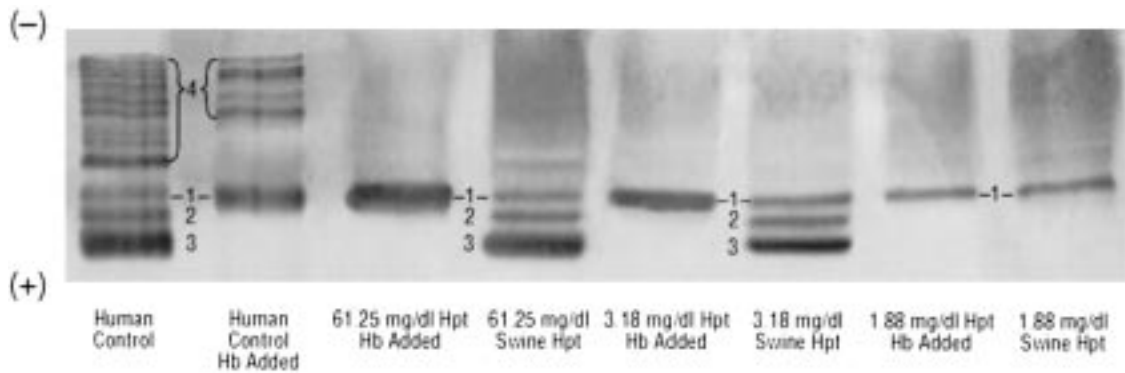


Figure 1. Western blot of pooled swine and human control serum samples with goat anti-human Hpt antiserum followed by rabbit anti-goat IgG phosphatase-labeled conjugate. Equal aliquots of pooled and control serum were preincubated with a saturating concentration of human Hb. Pooled swine serum Hpt concentrations as determined by CHB assay are shown. Bands labeled 1 or 2 are presumed saturated Hpt-Hb or Hpt-Hb intermediate complex, respectively. Bands labeled 3 are unbound Hpt. Bands labeled 4 are likely human subtypes.

amounts of swine Hb made from lysed red blood cells to both pooled high- and low-HBC serum samples.

With immunoelectrophoresis, the pooled serum samples all showed single distinct bands of recognition with the anti-Hpt antiserum at levels of anodal migration comparable to the human control.

Three bands of identity were revealed on western blot with the pooled medium- and high-HBC serum to the anti-Hpt antibody. Incubation of the serum samples with excess amounts of Hb prior to electrophoresis reduced the 3 bands identified in pooled medium- and high-HBC samples to 1 band similar to that found in both the pooled low-HBC serum with and without Hb added. The pooled low-HBC sample displayed only 1 band of identity, whereas the human serum control showed multiple bands of recognition. The human serum sample incubated with Hb also displayed a reduced number of bands (Fig. 1).

A strong positive correlation ($y = 0.953x + 2.65$, $r = 0.816$) was shown between the CHB assay and the immunoturbidimetric assay and between the 2 models of analyzer ($r = 0.960$). The immunoturbidimetric assay run on the newer model was linear upon serial dilution of pooled high-HBC serum with both pooled low-HBC serum ($r^2 = 0.989$) and saline ($r^2 = 0.986$). On the older model, the assay was linear when pooled low-HBC serum was diluted with saline ($r^2 = 0.994$); however, linearity was lost with the pooled high-HBC serum when values reached above 50 mg/dl. Within-run and between-run CVs were all $<9.0\%$ for both high- and low-HBC pooled serum on both instruments. Hemolysis caused an apparent decrease in Hpt values with increasing concentrations.

Both immunoelectrophoresis and western blot analysis confirmed that the anti-Hpt antiserum used in this immunoassay is monospecific for swine Hpt. The increased number of bands in the western blot is most likely due to the greater sensitivity of this assay over the immunoelectrophoresis procedure. The pattern of bands found on the western blot was similar to that observed using both canine and equine serum and this assay technique.¹³ The 3 bands observed on the western blot represent different levels of saturation of Hpt with Hb. Fully saturated Hpt-Hb complex is

represented by the most anodal band, and a Hpt-Hb intermediate and free Hpt are represented by the middle band and the band with the least anodal migration, respectively. Once Hb is added in excess, all of the serum Hpt is fully saturated. The single band so produced corresponds to the band with the least anodal migration. The lack of a similar band pattern in the pooled low-HBC serum sample is most likely an effect of the lower concentration and insufficient sensitivity of the western blot technique. The human control acts as previously described,¹³ with the numerous bands corresponding to the variety of Hpt and Hb subtypes found in human serum.

The results of this study compare favorably to the results of other studies that used other automated assays.^{7,11} The advantages of this assay over others include the fact that it is available as a kit sold commercially and it is easily adapted to different models of chemistry analyzers. The ability to use the same standards and antiserum for many different species negates the need for production and storage of several species-specific standards and antisera. The values obtained can be expressed as human Hpt equivalents, which also negates the need for unit conversion between species. Interference from hemolysis decreased the apparent recovery of Hpt in the serum samples, as was previously found using canine and equine serum.¹³ Although these studies show that Hb binding to Hpt does not completely diminish the ability of the anti-Hpt antibody to bind with Hpt, the matrix so formed must be altered. Hb binds to the β -chain of the Hpt molecule, masking some of its antigenic determinants.^{8,10} When the β -chain is bound, the α -chain retains antigenic properties.⁷ The Hpt-Hb complex and Hpt-Hb intermediate are not detectably affected, when examined by immunoelectrophoresis or western blot analysis. The binding of Hb to Hpt may affect the tertiary structure and alter Hpt or sterically hinder antigenic epitopes, resulting in decreased lattice formation. Therefore, any sample with visible hemolysis may produce abnormal Hpt values, which warrant caution in interpretation.

Slight modifications to the manufacturer's instructions are necessary for analyzing swine serum for Hpt and to ensure optimal assay performance. Although normal reference rang-

es were not determined in this study, swine serum contains overall lower total serum Hpt concentrations than does the serum of other species examined.^{2,4-6} Dilution of the standards and control facilitates a more accurate measure of swine serum Hpt concentration. According to the manufacturer's protocol, serum samples should be diluted 1:11 when adapted to the older model instrument. With slight adjustments to instrument parameters, an undiluted serum sample may be used, which alleviates an added step of diluting samples. However, a disadvantage to using an undiluted serum sample is loss of linearity at serum Hpt values >50 mg/dl. This is most likely a prozone effect on the assay, and any serum sample identified with a value >50 mg/dl when analyzed on the older model should be diluted into a working range.

This human Hpt immunoturbidimetric assay is valid for the measurement of serum Hpt in swine and may be used routinely in a swine production unit. However, future clinical studies will be necessary to assess the economic impact such assays may have on swine production.

Acknowledgement. We thank the Incstar Corporation for the gift of assay kits used in this study.

Sources and manufacturers

- a. Incstar Corp., Stillwater, MN.
- b. Ciba Corning Diagnostic Corp., Palo Alto, CA.
- c. Millipore Corp., Bedford, MA.
- d. Kirkgaard & Perry Laboratories, Gaithersburg, MD.
- e. Boehringer-Mannheim Corp., Indianapolis, IN.

References

1. Conner JG, Eckersall PD: 1988, Acute phase response in the dog following surgical trauma. *Res Vet Sci* 45:107-110.
2. Eckersall PD, Saini PK, McComb C: 1996, The acute phase response of acid soluble glycoprotein, α_1 -acid glycoprotein, ceruloplasmin, haptoglobin and C-reactive protein, in the pig. *Vet Immunol Immunopathol* 51:377-385.
3. Elson EC: 1974, Quantitative determination of serum haptoglobin. *Am J Clin Pathol* 63:655-663.
4. Eurell TE, Bane DP, Hall WF, Schaeffer DJ: 1992, Serum haptoglobin concentration as an indicator of weight gain in pigs. *Can J Vet Res* 56:6-9.
5. Francisco CJ, Bane DP, Weigel RM, Unverzagt L: 1996, The influence of pen density, weaning age, and feeder space on serum haptoglobin concentration in young growing swine. *Swine Health Prod* 4:67-71.
6. Hall WF, Eurell TE, Hansen RD, Herr LG: 1992, Serum haptoglobin concentration in swine naturally or experimentally infected with *Actinobacillus pleuropneumoniae*. *J Am Vet Med Assoc* 201:1730-1733.
7. Kent JE, Goodall J: 1991, Assessment of an immunoturbidimetric method for measuring equine serum haptoglobin concentrations. *Equine Vet J* 23:59-66.
8. Krawczyk E, Dobryczycka W: 1980, Properties of the interspecies hybrids between haptoglobin α and β subunits. *Acta Biochim Pol* 27:335-343.
9. Lampreave F, Gonzalez-Ramon N, Martinez-Ayensa S, et al.: 1994, Characterization of the acute phase serum protein response in pigs. *Electrophoresis* 15:672-676.
10. Shim BS, Yoon CS, Oh SK, et al.: 1971, Studies on swine and canine serum haptoglobins. *Biochim Biophys Acta* 243:126-136.
11. Skinner JG, Roberts L: 1994, Haptoglobin as an indicator of infection in sheep. *Vet Rec* 134:33-36.
12. Spooner RL, Miller JK: 1971, The measurement of haemoglobin reactive protein in ruminants as an aid to the diagnosis of acute inflammation. *Vet Rec* 88:2-4.
13. Wiedmeyer CE, Solter PF: 1996, Validation of human haptoglobin immunoturbidimetric assay for detection of haptoglobin in equine and canine serum and plasma. *Vet Clin Pathol* 25:141-146.

J Vet Diagn Invest 11:297-299 (1999)

Avian sexing: an optimized protocol using polymerase chain reaction-single-strand conformation polymorphism

O. Cortés, A. Barroso, S. Dunner

Determination of sex is of considerable importance for the understanding of numerous features of behavior, evolutionary ecology, genetics, and evolution² and in disease susceptibility.¹⁵ In many cases, sex is difficult or impossible to assess on the basis of phenotype. In birds, where females are heterogametic, the sex determination at the DNA level exploits the use of molecular W-linked markers. A methodology to identify molecular genetic markers for determination of sex using randomly amplified DNA fragments has been

described.⁸ This technology was followed by the isolation of a highly conserved gene, the chromodomain-helicase-DNA-binding gene (CHD), which exists in 2 copies in the avian genome. One of the genes (CHD-NW), first thought to be autosomal, is probably located on the Z chromosome,^{5,7} and the other is W-linked.⁹ The amplification of the CHD gene using a pair of polymerase chain reaction (PCR) primers developed previously⁹ and digestion with a restriction enzyme, which has to be modified depending on the species studied, is however not always satisfactory.¹⁰ An alternative strategy is to design PCR primer pairs amplifying fragments of appreciably different size from the CHD-W and CHD-NW genes, circumventing the need for restriction digestion

From the Laboratorio de Genética Molecular, Departamento de Producción Animal, Facultad de Veterinaria, 28040 Madrid, Spain.
Received for publication April 3, 1998.