Performance Evaluation of a Polyclonal Based Fecal Alpha-1-Antitrypsin ELISA

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ABSTRACT

Background: Alpha-1-antitrypsin (A1A) is a serum protease inhibitor that inhibits a wide range of proteases. A1A resists degradation by digestive enzymes and therefore, can be used to detect the presence of serum proteins in the gastrointestinal tract. The primary causes of protein-losing enteropathy can be divided into erosive gastrointestinal disorders, non-erosive gastrointestinal disorders, and disorders involving increased central venous pressure or mesenteric lymphatic obstruction. The diagnosis of protein-losing enteropathy is most commonly based on the determination of fecal A1A clearance. The purpose of this study was to assess the performance characteristics of a polyclonal antibody based A1A ELISA for the measurement of A1A in stool and to determine reference limits for A1A in stool and A1A clearance.

Methods: Stool samples included deidentified residual random stool specimens sent to ARUP Laboratories for routine testing, and timed stool specimens and paired serum obtained from healthy volunteers. A1A was extracted from stool and measured using the ImmuChrom Human A1A ELISA (ImmuChrom, GmbH, Heppenheim, Germany) according to the manufacturer's instructions. Serum A1A was measured using the Tina-quant® A1A assay (ver.2) on a cobas® 8000 modular analyzer (Roche Diagnostics, Indianapolis, IN). The performance characteristics evaluated for the stool ELISA were analytical sensitivity, linearity, accuracy, precision, and A1A stability in stool. The University of Utah's Internal Review Board approved the project.

Results: The analytical sensitivity was 0.14 ng/mL (0.002 mg/g stool) determined from 23 replicates of the sample diluent (mean + 3SD). Linearity was evaluated using diluted stool extracts with elevated A1A concentrations (range, 1.5 – 90.0 ng/mL). Linear regression produced results of y = 1.02x - 3.15 (r^2 = 0.986). Accuracy was determined from analyte recovery studies by adding sera of known A1A concentration into previously measured stool extracts. Recovery (measured ng A1A / expected ng A1A) ranged from 95.2 to 118.4%. Precision was determined from ten stool extracts obtained from each of two random stool specimens, with one extract analyzed in five replicates each day for ten days. Repeatability and within-laboratory CVs were 5.4 and 6.5% at 17.3 ng/mL (0.21 mg/g) and 13.8 and 14.5% at 66.9 ng/mL (0.84 mg/g), respectively. A1A was stable in stool for a minimum of 2 days, 7 days and 3 months at room temperature, 4 – 8 °C and -20 °C, respectively. A1A measured in timed stool obtained from 45 healthy volunteers (21 males, 24 females, ages 21 – 61) ranged from <0.002 to 0.59 mg/g stool. Using a robust skewed method, the reference limits for A1A in stool and A1A clearance were ≤0.47 mg/g and ≤45 mL/day, respectively.

Conclusions: The ImmuChrom Human A1A ELISA demonstrates acceptable performance for quantifying A1A in stool. The assay can also be used in conjunction with the Roche Diagnostics Tina-quant A1A (ver.2) assay for assessing A1A clearance.

INTRODUCTION

Alpha-1-Antitrypsin (A1A) is a serum protease inhibitor that inhibits a wide range of proteases. Because A1A resists degradation by digestive enzymes, the measurement of A1A in stool can be used to detect the presence of serum proteins in the gastrointestinal tract. The major causes of protein-losing enteropathy can be divided into: Erosive gastrointestinal disorders, non-erosive gastrointestinal disorders, and disorders involving increased central venous pressure or mesenteric lymphatic obstruction. Among such disorders are enteritis, Crohn's disease, ulcerative colitis and celiac disease.

The recommended testing for diagnosing protein-losing enteropathy is A1A clearance which is calculated using the A1A concentrations measured in serum and a timed stool collection. Although A1A clearance is the preferred method for protein-losing enteropathy, the A1A concentration in random stool can be used as an alternative in the absence of a timed stool specimen.

The ImmuChrom Human A1A ELISA test kit is a 96-well microtiter plate formatted immunoassay designed for the quantitative measurement of human A1A according to the "sandwich" principle. The procedure begins with extraction of A1A protein from 100 mg of stool with 5.0 mL of wash buffer. The extracts are then diluted 250-fold. Standards, controls and the patient unknowns (diluted extracts) are then added to designated wells and incubated. During incubation, A1A in the samples, standards and controls bind to antibodies coated to the microtiter plate. After a wash step to remove unbound materials, a second antibody conjugated to horseradish peroxidase (HRP) is added. This secondary antibody binds to the captured A1A protein, thus, providing a means of detection and quantification of A1A in the samples. After a second incubation period and washing, a chromogenic substrate (tetramethylbenzidine) is added. During this final incubation step, color is produced by HRP turnover of the substrate generating a direct relationship between the color intensity and the A1A concentration of each standard and sample. An acidic stopping solution is then added to terminate the reaction. The color intensity (or optical density, OD) of each sample is then measured spectrophotometrically at 450 nm (against a reference of 620 nm). Lastly, a calibration curve is constructed (OD vs. concentration) from the five standards of assigned A1A concentrations and the control and unknown concentrations calculated from the curve. The patient unknown results, expressed in units of ng/mL, are then multiplied by a factor of 0.0125 to accommodate the extraction and ELISA dilution factors, and convert results to the final reported units of mg/g stool.

The Roche cobas Tina-quant A1A ver.2 test is an FDA cleared automated immunoturbidimetric assay. A1A present in human serum forms a precipitate with a specific antiserum. Quantification is then determined turbidimetrically and reported in units of mg/dL.

A1A clearance is calculated using the fecal A1A concentration, the grams of stool collected, the time interval of the collection and the serum A1A concentration from blood drawn during the stool collection period. Final results are reported in units of mL/day.

MATERIALS AND METHODS

- ImmuChrom Human Alpha-1-Antitrypsin ELISA kits (ImmuChrom, GmbH, Heppenheim, Germany) were purchased from BioVendor, LLC (Asheville, NC).
- Tina-quant® α1-Antitrypsin ver.2 assay kits were acquired from Roche Diagnostics (Indianapolis, IN).
- SPECTRAmax® PLUS plate reader was manufactured by Molecular Devices Corp. (Sunnyvale, CA) and controlled using Molecular Devices Corp. SoftMax Pro® software.
- Cobas® 8000 c702 analyzer was obtained through Roche Diagnostics (Indianapolis, IN).
- Residual random stool specimens, sent to ARUP for routine testing, were deidentified using University of Utah Internal Review Board approved protocols.
- Reference interval timed stool and serum specimens (62 males, 62 females; 19 to 61 years) were obtained from healthy volunteers under a University of Utah Internal Review Board approved protocol.
- Stool samples, extracts and serum samples were stored short term (<2 days) at 2 – 8 °C and for longer periods at -20 °C.
- Fecal A1A was extracted and measured according to the ELISA kit manufacturer's protocol.
- Serum A1A was measured using the cobas analyzer by a previously validated procedure routinely used for assessing A1A deficiency.
- Data analysis was performed using StatisPro™ method evaluation software (Clinical and Laboratory Standards Institute®, Wayne, PA), Microsoft® Office Excel® (Microsoft Corporation, Redmond, WA) and GraphPad Prism® (GraphPad Software Inc., La Jolla, CA).
- Incorporating the 50-fold dilution for extraction (assumption: 1 g stool = 1 mL) and the 250-fold dilution of the extract before testing, fecal A1A results were converted from ng/mL units to mg/g units as follows:

$$\frac{\text{ng}}{\text{mL}} \times 50 \times 250 \times \frac{1\,\mu\text{g}}{1000\,\text{ng}} \times \frac{1\,\text{mg}}{1000\,\mu\text{g}} = 0.0125 \frac{\text{mg}}{\text{mL}} = \frac{\text{mg}}{\text{g}}$$

Therefore,

$$\text{Result in } \frac{\text{ng}}{\text{mL}} \times 0.0125 = \text{Result in } \frac{\text{mg}}{\text{g}}$$

- A1A clearance (mL/day) was calculated using the following results and equation: Stool mass (g), collection interval (day), fecal A1A (mg/g), serum A1A (mg/dL).

$$\text{A1A Clearance (mL/day)} = \frac{\text{g stool}}{\text{days of collection}} \times \frac{\text{mg stool A1A}}{\text{g}} \times \frac{\text{dL}}{\text{mg serum A1A}} \times \frac{100\,\text{mL}}{\text{dL}}$$

- Unless noted otherwise, reported fecal A1A results are the means of duplicate measurements. Serum results are from singlet measurements.

RESULTS

- The A1A stool ELISA's limit of blank was 0.14 ng/mL, equivalent to 0.002 mg/g stool, and well below the claim by the test kit manufacturer of 1.5 ng/mL (0.02 mg/g stool). (23 determinations of the sample diluent, mean + 3SD).
- **Figure 1.** Linearity was found acceptable throughout the procedure's claimed analytical measurement range of 1.5 – 90 ng/mL, with slope = 1.02 (95% confidence interval or CI = 0.920 – 1.128), intercept = -3.15 (95% CI = -7.701 – 1.337) and r^2 = 0.986.
- **Table 1.** Analyte recovery (measured ng A1A / expected ng A1A) ranged from 95.2 to 118.4%. The mean recovery was 107.7% (n = 12).
- **Table 2.** Imprecision, including the A1A extraction step for within-laboratory, was acceptable with repeatability and within-laboratory CVs of 5.4 and 6.5% at 17.3 ng/mL (0.21 mg/g) and 13.8 and 14.5% at 66.9 ng/mL (0.84 mg/g), respectively.
- **Table 3.** A1A was stable in stool for minimums of 2 days at room temperature, 7 days refrigerated and 14 days frozen. In stool extracts, A1A was stable for a minimum of 6 months frozen.
- **Figure 2A.** Updated reference interval studies (n increased to 124 since abstract submission), produced a fecal A1A reference limit of ≤0.48 mg/g stool (nonparametric analysis, 95th percentile; 95% CI = 0.370 – 0.670 mg/g). Results ranged from <0.02 – 89 mg/g. A difference between males and females was not evident (p = 0.646). A correlation between fecal A1A vs. age was also insignificant (p = 0.739).
- **Figure 2B.** The updated A1A clearance reference interval study generated a limit of 49 mL/day (nonparametric analysis, 95th percentile; 95% CI = 36.1 – 72.8 mL/day). Results ranged from 0 – 80 mL/day. Serum A1A ranged 71 – 225 ng/dL. (Previously established serum reference interval: 100 – 200 ng/dL.) The variance between males and females was insignificant (p = 0.394). A correlation between A1A clearance vs. age was also not apparent (p = 0.158).

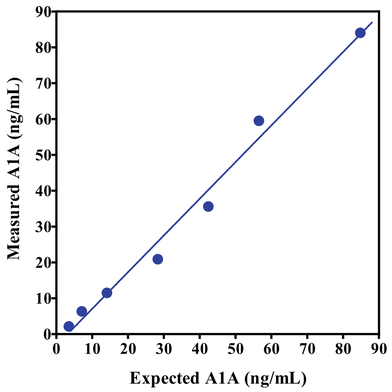

Figure 1. ImmuChrom Alpha-1-Antitrypsin ELISA Linearity. Linear regression of measured vs. expected results from a highly concentrated A1A stool extract diluted to seven different concentrations. Slope = 1.02, intercept = -3.15 and r^2 = 0.986. Analytical measurement range: 1.5 – 90 ng/mL (0.02 – 1.13 mg/g).

Table 1. Percent recovery: ImmuChrom Alpha-1-Antitrypsin ELISA.

Sample ID[a]	ng A1A in Sample: Expected	Measured	% Recovery[b]
1	17.6	18.8	106.7
2	22.0	25.0	113.4
3	26.5	30.1	113.6
4	6.7	7.0	104.5
5	8.9	9.3	104.1
6	13.4	14.9	111.2
7	13.5	14.6	107.9
8	17.9	19.0	105.7
9	22.4	22.2	99.3
10	40.7	38.7	95.2
11	45.1	50.8	112.5
12	54.0	63.9	118.4

[a]Serum sample of known A1A concentration spiked into stool extracts of known A1A concentrations.

[b]Measured/Expected. Mean recovery: 107.7%.

Table 2. Imprecision: ImmuChrom Alpha-1-Antitrypsin ELISA.*

	Level I	Level II
Mean (ng/mL)	17.3	66.9
Repeatability SD	0.93	9.20
Repeatability CV	5.4%	13.8%
Within-Laboratory SD	1.12	9.69
Within-Laboratory CV	6.5%	14.5%

*One run per day for ten days, five replicates per run per concentration level. Each run includes the stool extraction step.

Table 3. Alpha-1-antitrypsin analyte stability in stool and stool extracts.

Stability in Stool: Room Temperature			Refrigerated, 4 – 8 °C			Frozen, -20 °C			Stability in Stool Extracts: Frozen, -20 °C		
	A1A (mg/g)			A1A (mg/g)			A1A (mg/g)			A1A (mg/g)	
Days	Level I	Level II	Days	Level I	Level II	Months	Level I	Level II	Months	Level I	Level II
0	0.22	0.84	0	0.21	0.36	0	0.22	0.84	0	0.09	0.18
2	0.21	1.08	1	0.20	0.39	1	0.24	0.87	2	0.09	0.19
			2	0.19	0.34	2	0.24	0.88	6	0.10	0.19
			3	0.18	0.33	3	0.23	0.84			
			7	0.17	0.35						
Mean	0.22	0.96	**Mean**	0.19	0.35	**Mean**	0.23	0.86	**Mean**	0.09	0.19
SD	0.007	0.170	**SD**	0.016	0.023	**SD**	0.010	0.021	**SD**	0.006	0.006
CV	3.3%	17.7%	**CV**	8.3%	6.5%	**CV**	4.1%	2.4%	**CV**	6.2%	3.1%

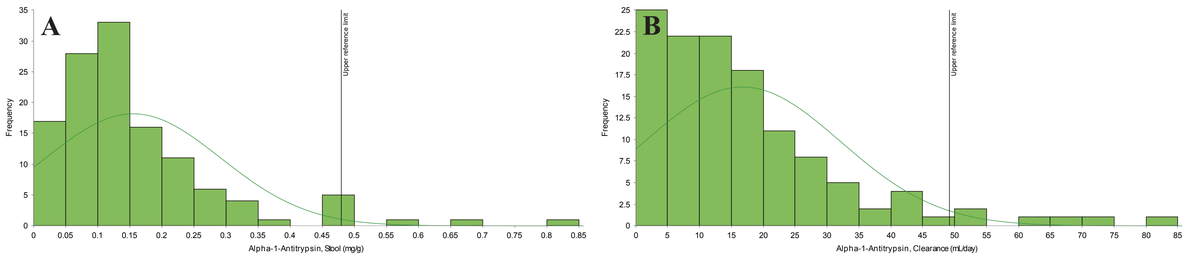

Figure 2. Fecal A1A and A1A Clearance Reference Intervals. Timed stool and serum A1A measured in 124 healthy volunteers (62 males, 62 females, ages 19 – 61 years). Reference interval limits calculated by nonparametric analysis at the 95th percentile. (A), a fecal A1A reference interval limit of ≤0.48 mg/g established (95% CI = 0.370 – 0.670 mg/g). (B), for A1A clearance, a reference interval limit of 49 mL/day determined (95% CI = 36.1 – 72.8 mL/day).

CONCLUSIONS

- The ImmuChrom Alpha-1-Antitrypsin ELISA demonstrates acceptable performance for quantifying A1A in human stool in both random and timed specimens.
- Timed stool A1A results measured using the ImmuChrom A1A ELISA, and serum A1A results determined by the Roche Diagnostics cobas Tina-quant α1-Antitrypsin ver.2 assay, can be combined for assessing A1A clearance in human subjects.
- Upper reference interval limits of ≤0.48 mg/g stool and 49 mL/day were established for fecal A1A and A1A clearance, respectively.

ACKNOWLEDGMENTS

We would like to thank Kristin Case, Technical Supervisor and Kisha Diamse for help with specimen collection and the recruitment of healthy volunteers. We also thank Jonathan Genzen, Medical Director, Sara Wyness and the ARUP Automated Core Laboratory for serum A1A testing.

Financial support provided by the ARUP Institute for Clinical and Experimental Pathology.

REFERENCES

- Gettins PG. Serpin structure, mechanism, and function. Chem Rev 2002;102:4751–804.
- Florent C, L'Hirondel C, Desmazures C, Aymes C, Bernier JJ. Intestinal Clearance of alpha 1-antitrypsin. A sensitive method for the detection of protein losing enteropathy. Gastroenterology 1981;81:777–80.
- Hill RE, Hercz A, Corey ML, Gilday DL, Hamilton JR. Fecal clearance of alpha 1-antitrypsin: a reliable measure of enteric protein loss in children. J Pediatr 1981;99:416-8.
- Greenwald D. Protein-Losing Gastroenteropathy. In: Feldman M, Friedman LS, Brandt LJ, Sleisinger MH, eds. Sleisenger & Fordtran's Gastrointestinal and Liver Disease. 8th Ed. Philadelphia: Saunders 2006:557–63.
- Grill BB, Hillemeier AC, Gryboski JD. Fecal alpha 1-antitrypsin clearance in patients with inflammatory bowel disease. J Pediatr Gastroenterol Nutr 1984;3:56-61.
- Karbach U, Ewe K. Enteric protein loss in various gastrointestinal diseases determined by intestinal alpha 1-antitrypsin clearance. Z Gastroenterol 1989;27:362–5.
- Bai JC, Sambuelli A, Niveloni S, Sugai E, Mazure R, Kogan Z, Pedreira S, Boerr L. Alpha 1-antitrypsin clearance as an aid in the management of patients with celiac disease. Am J Gastroenterol 1991;86:986–91.
- Human Alpha-1-Antitrypsin ELISA [Package insert]. Version 08 311007.
- Tina-quant α-1-Antitrypsin ver.2 [Package insert]. December 2012.