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Detection and diagnostic value of urine leucine-rich alpha-2-glycoprotein (LRG) in children with suspected acute appendicitis

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Abstract

Objective—Previously, we used a proteomics approach for the discovery of new diagnostic markers of acute appendicitis (AA) and identified LRG that was elevated in the urine of children with AA and enriched in diseased appendices. Here, we sought to evaluate the diagnostic utility of enzyme-linked immunoassay (ELISA) of urine LRG in a blinded, prospective, cohort study of children being evaluated for acute abdominal pain.

Methods—Urine LRG concentration was measured using a commercially available LRG ELISA, and selected ion monitoring (SIM) mass spectrometry (MS). Urine LRG test performance was evaluated blindly against the pathologic diagnosis and histologic grade of appendicitis.

Results—Urine LRG was measured in 49 patients. Mean urine LRG concentration measured using commercial LRG ELISA was significantly elevated in patients with AA, but exhibited an interference effect. Direct measurements using SIM MS demonstrated that LRG was elevated more than 100-fold in patients with AA as compared to those without, with the receiver operating characteristic area under the curve of 0.98 (95% CI = 0.96-1.0). Among patients with AA, elevations of urine LRG measured using ELISA and SIM MS correlated with the histologic severity of appendicitis.

Conclusion—Urine LRG ELISA allows for discrimination between patients with and without AA, but exhibits limited accuracy due to immunoassay interference. Direct measurements of urine LRG using SIM MS demonstrate superior diagnostic performance. Development of a clinical-grade urine LRG assay is needed to advance the diagnostic accuracy of clinical evaluations of appendicitis.

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Conflict of interest The authors declare no conflicts of interests.

Introduction

Acute appendicitis is the most common surgical emergency of children and delays in diagnosis are associated with increased appendiceal rupture, morbidity and cost.¹ Despite attempts to develop clinical scores to guide clinical management of children with suspected appendicitis, their performance has been limited by classifying many children with intermediate risk.² Use of computed tomography (CT) and ultrasound has led to significant reduction in unnecessary appendectomies.³ CT has superior diagnostic performance compared to ultrasound, but concerns of long-term cancer risk has led to recommendations to limit its use.⁴ Thus, diagnosing appendicitis remains a major challenge and improved techniques to discriminate appendicitis from other mimicking conditions are urgently needed.

Previously, we used a discovery-based mass spectrometry proteomic approach to identify specific markers of appendiceal inflammation, including leucine-rich alpha-2-glycoprotein (LRG).⁵ LRG is enriched in diseased appendices and elevated in the urine of patients with appendicitis, including patients without radiographic evidence of appendicitis. The translational significance of LRG is further supported by its roles in the activation and chemotaxis of neutrophils, whereby it may underlie a principal pathway of appendiceal inflammation.⁶ Recently, enzyme-linked immunoassays were developed for LRG, suggesting that an analytical clinical laboratory test for LRG may be possible. However, the diagnostic performance of LRG ELISA for the detection of urine LRG in patients with acute appendicitis is not known.

Here, we investigated the diagnostic performance of a commercially available LRG ELISA. To judge the test performance of LRG, we developed a direct assay of specific LRG peptides using selected ion monitoring (SIM) mass spectrometry (MS).

Methods

This study was conducted at a tertiary care pediatric emergency department and approved by the Children's Hospital Boston Committee on Clinical Investigation. A convenience sample of patients younger than 18 years of age being evaluated for possible appendicitis were enrolled through a standard consent and assent process. Surgical consultation or advanced imaging for the primary evaluation of appendicitis was required for patients to be considered for study enrollment. Patients were excluded if they had pre-existing autoimmune, neoplastic, renal or urologic disease or were pregnant. Complete blood counts (including absolute neutrophil counts, ANC) were obtained as part of routine care. Serum C-reactive protein (CRP) was often obtained as part of routine care and when not, CRP was ordered as part of study protocol (but not available to clinical team under those circumstances). Urine was collected as clean-catch, midstream samples and stored at -80 °C within 6 hours of collection. Urine specimens were labeled with a study number such that all analysis was blinded.

Final diagnosis was determined by the presence or absence of appendicitis on gross and histologic examination. All disease assignments were confirmed independently upon blinded histologic review of appendectomy specimens. Patients with perforated appendicitis that underwent interval appendectomies were not included in the histologic review. Histologic severity of appendicitis was accomplished by classifying the specimens as having no inflammatory features (normal), foci of neutrophilic infiltration in wall or mucosa (focal), scattered transmural infiltration (mild), dense transmural infiltration with tissue distortion (moderate), dense transmural infiltration with tissue necrosis or wall perforation (severe). For patients who did not undergo appendectomies, the outcome was confirmed by telephone

6-8 weeks after evaluation using scripted questions. Clinical and laboratory data was tracked using standardized case report forms.

LRG ELISA was obtained from IBL International (Toronto, Canada). For serial dilutions, urine specimens were diluted with the ELISA sample buffer. Testing of immunoassay interference was done using SuperBlock TBS blocking buffer (Pierce, Thermo Scientific). Adjustment for immunoassay interference were done by measuring urine specimens diluted 5, 10, 100, 1,000, 10,000, and 50,000-fold, and discounting measurements diluted less than 100-fold which exhibited paradoxical increases in apparent LRG concentration with increasing dilution. For LRG SIM MS assay, we utilized the filter-aided protein purification (FASP) protocol with the following modifications⁷: one ml urine aliquots were loaded on Microcon YM-10 concentrators (Millipore) in the presence of 8 M urea and 100 mM dithiothreitol. Proteins were washed twice with 0.3 ml of urea buffer, alkylated with iodoacetamide, washed three times with 0.3 ml ammonium bicarbonate buffer, and digested for 16 hours with 1 µg of trypsin at 37 °C. Tryptic peptides were purified using reversed phase affinity chromatography and resolved using liquid chromatography (LC) tandem mass spectrometry with a microscale capillary HPLC system (Famos and Agilent) coupled to a hybrid quadrupole-time of flight mass spectrometer (QStar XL, AB/Sciex). LRG peptides ALGHLDLSG NR (m/z 384.88) and VAAGAFQGLR (m/z 495.28) were targeted for fragmentation to unambiguously confirm the peptide identity. For the subsequent quantification, extracted ion chromatograms were generated for m/z-window of 0.05 Da. The peptide abundances were calculated by integrating the area under the peaks using MultiQuant (version 2.0.2, AB/Sciex). Receiver operating characteristics were calculated using standard methods (Origin, version 8, OriginLab). Statistical significance of measured differences was calculated using the Wilcoxon rank-sum test, as implemented in STATA (version 10.1, StataCorp). All statistical tests were two-tailed.

Results

We enrolled 49 patients who underwent evaluation for possible appendicitis. The mean ($\pm$ standard deviation) age of our cohort was 10.9 ($\pm$ 4.3) years and 53% were male. Their presenting signs and symptoms are described in Table 1. Twenty four patients received a final diagnosis of appendicitis, 79% of whom had moderate or severe appendicitis on histologic evaluation. Three patients (12%) with confirmed appendicitis were found to have no radiographic evidence of appendicitis upon CT or US imaging. Four patients (16%) who were deemed to have radiographic evidence of appendicitis and received a pre-operative diagnosis of appendicitis were found to have no gross or histologic evidence of appendicitis upon undergoing appendectomy. The remaining patients without appendicitis were found to have non-specific acute abdominal pain, as confirmed 6-8 weeks after evaluation.

Relatively high concentrations of LRG in the urine of patients with appendicitis required that urine specimens be diluted prior to ELISA measurement. We found that for some specimens repeated measurements using serially diluted specimens were highly reproducible with relatively low variability in apparent urine LRG concentration (green, Fig. 1A). In contrast, other specimens demonstrated a paradoxical increase in the apparent LRG concentration with increasing urine dilution (red, Fig. 1A), indicative of an immunoassay interference effect. This is most often due to the presence of substances that interfere with the adsorption of LRG onto the capture antibody or its detection. Dilution of such interfering substances increases the availability of LRG for detection and increases its apparent concentration. We found that for a subset of specimens, dilution by at least 100-fold allowed for accurate measurement of urine LRG, with relatively low variability in apparent urine LRG concentration measured at higher dilution (red, Fig. 1A). Attempts to mitigate the interference effect by enhanced blocking of ELISA plates or varying ELISA pH was not

effective (Appendix, available online at www.annemergmed.com, Supp. Fig. 1). Also, we found no differences in apparent urine LRG concentration between freshly collected and frozen urine specimens (Appendix, available online at www.annemergmed.com, Supp. Fig. 2). Thus, serial dilution is able to overcome the LRG ELISA interference effect in the urine of some but not all patients with suspected appendicitis (Fig. 1A).

Thus, we proceeded to investigate whether interference-adjusted urine LRG ELISA measurements can be used to discriminate between patients with and without appendicitis. We found that the interference-adjusted urine LRG concentration was significantly elevated in patients with appendicitis (3.9 $\mu\text{g/ml}$ [0.9, 19.3], median [IQR]) as compared to those without 0.3 $\mu\text{g/ml}$ [0.1, 0.8]; median difference: 3.3 $\mu\text{g/ml}$, 95% CI [0.5, 17.5], Appendix, available online at www.annemergmed.com, Supp. Fig. 3). Consistent with prior observations, we also found that urine LRG levels correlated with the histologic severity of appendicitis (Fig. 1B). However, this analysis also revealed an overlap in the distributions of urine LRG ELISA concentrations between patients with mild appendicitis and those without (Fig. 1B).

We used quantitative mass spectrometry to measure urine LRG directly, without relying on antibodies that are subject to potential immunoassay interference. We monitored two LRG-derived tryptic peptides ALGHLDSLGNR and VAAGAFQGLR. These peptides were chosen because they are uniquely present in LRG, can be efficiently generated using trypsin proteolysis, and are readily measured using selected ion monitoring mass spectrometry. Using this SIM MS assay, we were able to robustly detect and quantify LRG in clinical urine specimens (Appendix, available online at www.annemergmed.com, Supp. Fig. 4). These direct urine LRG measurements revealed a substantially greater discrimination between patients with appendicitis and those without (Fig. 1C), as compared to the LRG ELISA measurements that were limited by immunoassay interference (Fig. 1B). We observed a statistically significant increase in SIM MS urine LRG levels in patients with severe as compared to moderate appendicitis ($p = 0.06$), but were not able to assess the correlation of SIM MS LRG with histologically less severe disease due to the low number of patients (only 1 patient with focal appendicitis).

Importantly, urine LRG exhibited excellent diagnostic performance as assessed using receiver operating characteristic analysis (Fig. 2). In agreement with prior findings, direct measurements of urine LRG had superior diagnostic performance with the area under the curve (AUC) of 0.98 and 0.99 for the two SIM MS assays (95% CI = 0.96-1.0, Supp. Fig. 5, Table 2). In contrast, currently used appendicitis diagnostic tests such as peripheral blood absolute neutrophil count (ANC) and serum C-reactive protein (CRP) exhibited AUC values of 0.73 and 0.76, respectively (Supp. Fig. 5, Table 2). As evidenced by the immunoassay interference effect that limits the accuracy of LRG measurements in patients with high LRG, urine LRG ELISA had a diminished diagnostic performance with the AUC value of 0.80 as compared to direct measurements using SIM MS (Supp. Fig. 5, Table 2).

Discussion

Appendicitis remains a major diagnostic challenge for clinicians even with the use of CT and US imaging. Radiographic findings can often be indeterminate, and currently used laboratory diagnostic markers are largely part of the general acute-phase response that is not specific for the distinct immune mechanisms that characterize acute appendicitis.^{8,9} We recently identified LRG to be enriched in diseased appendices and elevated in the urine of patients with appendicitis, including patients without radiographic evidence of appendicitis.⁵ We were able to detect urine LRG using an immunoassay suggesting that clinical LRG

testing in the context of interventional clinical trials may be accomplished using conventional immunoassays such as ELISA.

Here, we investigated the performance of a commercially available LRG ELISA in a blinded, retrospective study of children suspected of acute appendicitis. We found that the use of this ELISA to measure urine LRG was complicated by the presence of an immunoassay interference effect. Though this immunoassay interference was partially mitigated by serial dilution of urine specimens (Fig. 1), it nonetheless caused the apparent LRG concentration to be lower in patients with appendicitis, and as a result significantly limited the diagnostic performance of this assay (Supp. Fig. 5, Table 2).

To allow direct measurements of LRG not susceptible to immunoassay artifacts, we developed a SIM MS assay using two independent LRG peptides that demonstrated improved measurement accuracy in clinical urine specimens. SIM MS urine LRG measurements exhibited superior diagnostic performance as compared to currently used diagnostic tests, including accurate diagnosis in patients with histologically confirmed appendicitis but no radiographic evidence of appendicitis upon CT or US imaging, as well as patients who underwent appendectomies but had no histologic evidence of appendicitis (Supp. Fig. 5, Table 2).

The use of urine LRG to augment current clinical decision rules may improve the accuracy and timeliness of diagnosing acute appendicitis. In principle, an inexpensive but accurate immunoassay could conceivably replace the use of advanced imaging in equivocal clinical presentations or could be performed in conjunction with serial examinations among patients with low probability of appendicitis but unexplained abdominal pain. Given its biologic function, LRG would likely be elevated in bacterial infections such as pneumonia, pelvic inflammatory disease, or pyelonephritis; therefore, use of LRG should be guided by the clinical suspicion of appendicitis and not by abdominal pain per se. Testing of LRG and other novel diagnostic markers in interventional clinical trials to improve the diagnosis of appendicitis is an important direction of future work.

Limitations

Our study of LRG as a urinary marker of acute appendicitis is limited by being conducted in a single institution with a small sample of children. For a patient to be studied, the attending physician must have suspected appendicitis. Since the threshold to consider appendicitis is clinician dependent, our study is subject to potential selection bias. The diagnostic value of LRG will require further study using a clinical-grade assay in multiple settings that include patients with a diverse range of causes of acute abdominal pain. Furthermore, although we have shown some association between LRG levels and histopathologic severity, the exact time between urine collection and operative care was not studied.

Conclusions

LRG is a promising urinary diagnostic marker for acute appendicitis. The commercially available LRG ELISA from IBL International does not appear to be suitable for clinical use in urine as a result of an immunoassay interference effect. This inadequate performance may be due to low affinity antibodies or possibly the presence of LRG mimicking epitopes by other urinary proteins. The SIM MS LRG assay presented here will be essential in developing improved antibodies for LRG immunoassays suitable for clinical measurements of urine LRG in patients with suspected appendicitis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A. Urine LRG ELISA is limited by immunoassay interference that can be partially mitigated by serial dilution. Apparent urine LRG concentration measured using commercial LRG ELISA as a function of urine dilution of 49 specimens. Most specimens exhibit increases in apparent LRG concentration with increasing dilution of urine, indicating the presence of a substance that interferes with the antibody adsorption and/or detection of urine LRG. This effect is absent in some specimens, with relatively constant LRG concentrations among repeated measurements of different dilutions (green). The immunoassay interference can be partially overcome by increasing urine dilution, with relatively constant LRG concentrations in specimens diluted by more than 100-fold (red). B. Urine LRG ELISA discriminates patients with appendicitis and correlates with disease severity. Boxplots of ELISA interference-adjusted urine LRG concentrations in patients without appendicitis (black), as compared to those with appendicitis with increasing histologic severity. LRG ELISA values in some patients with appendicitis overlap with those in patients without appendicitis or histologically normal appendices. C. Direct measurement of urine LRG using SIM MS overcomes immunoassay interference effects. SIM MS urine LRG levels as measured using area under the curve (AUC) for the extracted ion currents of LRG peptide VAAGAFQGLR in patients with histologically normal appendices, as compared to those with appendicitis with increasing histologic severity. Box center and ranges represent median and standard error values, respectively. Stars denote mean values for the two groups. Whiskers represent 95% confidence intervals.

Table 1

Presenting signs, symptoms and diagnostic studies of 49 patients with acute abdominal pain and suspected appendicitis.

	Final Diagnosis	
	Appendicitis	Non-appendicitis
Number	24	25
Gender (% male)	62	44
Age (years)	11.2 ± 5.1	10.5 ± 3.6
Duration of symptoms (hours)		
<12 hours (%)	30	45
13-23 hours (%)	30	17
24-35 hours (%)	18	21
36-48 hours (%)	18	13
48-72 hours (%)	4	4
Nausea or vomiting (%)	54	36
Fever (%)	46	52
Pain migration to RLQ (%)	62	28
Focal RLQ pain or tenderness (%)	83	48
Temperature at triage (° C)	37.5 ± 0.7	37.1 ± 0.9
Peripheral white blood cell count (K cells/mm ³)	15.7 ± 6.4	10.1 ± 4.7
Absolute neutrophil count (K cells/mm ³)	12.8 ± 6.1	7.3 ± 5.0
Serum C-reactive protein (mg/dL)	5.8 ± 4.5	0.42 ± 0.33
Histology		
Focal appendicitis (%)	4	N/A
Mild appendicitis (%)	17	N/A
Moderate appendicitis (%)	54	N/A
Severe appendicitis (%)	25	N/A
US imaging (%)	83	100
CT imaging (%)	21	65
US diagnosis of appendicitis (%)	63	12
CT diagnosis of appendicitis (%)	21	8

Values are reported as mean ± standard deviation, where appropriate. RLQ (right lower quadrant), US (ultrasound), CT (computed tomography).

Table 2

Diagnostic performance of markers of acute appendicitis

Marker	ROC AUC	AUC 95% Confidence Interval
ANC	0.73	0.59-0.87
CRP	0.76	0.54-0.98
LRG ELISA	0.80	0.67-0.92
LRG SIM MS1	0.98	0.96-1.0
LRG SIM MS2	0.99	0.96-1.0

ROC receiver operator characteristic; AUC area under curve; ANC absolute neutrophil count; CRP c-reactive protein; LRG leucine-rich alpha 2 glycoprotein; ELISA enzyme-linked immunosorbent assay; SIM selected ion monitoring; MS mass spectroscopy