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Sensitive Noninvasive Marker for the Diagnosis of Probable Bacterial or Viral Infection

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Urinary trypsin inhibitor (uTi) is a product of elastase-mediated degradation of interleukin-α-inhibitor (I-α-I). Its activity increases in the urine of patients with a malignancy, inflammation, or infection, or in late pregnancy. The objective of this study was to compare the sensitivity of uTi in urine with that of serum quantitative C-reactive protein (CRP) for diagnosing infection, as indicated by white cell response and clinical assessment. Ninety controls and 171 patients with various systemic infections were enrolled. We measured uTi enzymatically on a Cobas Fara (Roche Diagnostics). Patients were separated into bacterial, probable bacterial, viral, or probable viral groups based on the results of a complete blood count with differential (CBC), urinalysis (UA), and clinical assessment. In the bacterial (n=70) and control (n=90) groups, the uTi values (mean ± SE) were 25.3 ± 3.1 mg/L and 2.8 ± 0.8 mg/L, respectively. uTi (at 2.7 mg/L) had a diagnostic sensitivity of 91% and specificity of 82% (AUC=0.889), whereas CRP (at a cutoff of 10 mg/L) had a sensitivity and specificity of 82% and 96%, respectively (AUC=0.921). As a marker of infection (positive in both bacterial and viral groups), uTi had a sensitivity of 91% (AUC=0.884) vs. 89% (AUC=0.828) for CRP. Our data indicate that uTi has sufficient clinical sensitivity for screening systemic infections, and may have diagnostic value as a noninvasive test. J. Clin. Lab. Anal. 18:289–295, 2004. © 2004 Wiley-Liss, Inc.

Key words: C-reactive protein; urine; elastase; WBC; inflammation; screen

INTRODUCTION

When assessing patients with suspected infection, physicians usually rely on physical examinations, the patient's history, and several tests, such as complete blood count with differential (CBC), urinalysis (UA), and erythrocyte sedimentation rate (ESR) (Table 1). As an aid to diagnosing bacterial infection, quantitative C-reactive protein (CRP) has gained popularity and has been shown to be superior to the ESR (1). Additional biomarkers, such as IL-8 (2), procalcitonin (2,3), and human neutrophil lipocalin (4), have also been considered as potential tools for diagnosing bacterial infection and inflammation. Many of these tests are performed on blood. A rapid noninvasive method suitable for measurements in urine may be a valuable diagnostic tool for screening patients with suspected inflammation secondary to infection.

Interleukin-alpha-inhibitor (I-α-I) is a member of the Kunitz-type family of serine proteases, which are responsible for shutting down the activities of various enzymes, including trypsin (5). I-α-I is normally present in the serum as a pro-inhibitor, and lacks protease inhibitory activity. During inflammation, a rise in the white blood cell (WBC) count increases serum elastase activity, which allows I-α-I to be cleaved into its active inhibitory forms. These active inhibitors are indicators

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TABLE 1. Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
AUC	Area under curve
CBC	Complete blood count
CRP	C-reactive protein
EDTA	Ethylenediaminetetraacetic acid
ESR	Erythrocyte sedimentation rate
HIV	Human immunodeficiency virus
I-α-I	Inter-alpha-inhibitor
L-BAPNA	N_α-benzoyl-L-arginine p-nitroanilide
ROC	Receiver operating characteristic
UA	Urianalysis
uTi	Urinary trypsin inhibitor
WBC	White blood count

of positive acute phase response (6), and they play a role in protecting noninfected locations by suppressing serine protease activity. They are readily excreted into urine, leading to their distinction as urinary trypsin inhibitors (uTi's). The prevalent uTi forms include Bikunin (molecular weight = ≈30 kDa) and its Uristatin fragments, which lack the chondroitin sulfate chain. These fragments are generally <17 KDa, and all exhibit trypsin inhibitory activity (7,8).

uTi activity has been suggested as a marker of bacterial infection (9,10), acute and fulminant hepatitis (6), and inflammation (7). In addition to the above-mentioned conditions, uTi is increased in the urine of individuals under a variety of conditions, including malignancy (11), surgery (12), and pregnancy (13).

The purpose of this study was to compare the sensitivity and specificity of uTi (a noninvasive marker in urine) to those of serum CRP, using white cell response as a reference standard in combination with CBC, blood cultures, and other end-points for infection. Based on our data, uTi is a clinically sensitive marker of infection as indicated by white cell response and clinical assessment.

MATERIALS AND METHODS

To measure uTi, we used an enzymatic method previously described by Kuwajima et al. (14). Briefly, bovine pancreatic trypsin was used as the enzyme, and N_α-benzoyl-L-arginine p-nitroanilide (L-BAPNA) was used as the substrate. The trypsin and L-BAPNA were purchased from Sigma (St. Louis, MO), and the uTi standard (marketed as Uristatin, product code P205-1) was obtained from SciPac Ltd. (Sittingbourne, Kent, UK). In the enzymatic assay for trypsin, the product formation was monitored in a kinetic reaction at 405 nm every 15 sec by a Cobas Fara II centrifugal spectrophotometric analyzer (Roche Diagnostics, Indianapolis, IN). The concentration of uTi in the sample was determined by this instrument based on a four-point standard curve (0, 12.5, 25, and 50 mg/L).

All uTi measurements were performed in batches of 10, with both controls included in each run. The overall imprecision was assessed with the use of two controls at target values of 10 and 30 mg/L analyzed over 14 separate days. The coefficient of variation was 14% (9.93 ± 1.42 mg/L) for the low control, and 3.7% (32.45 ± 1.21 mg/L) for the high control. The purpose of our study was to compare the clinical sensitivities and specificities of uTi and CRP for detecting probable viral or bacterial infections as indicated by CBC or positive blood culture results. Therefore, in our hospital's clinical laboratory we performed assays for blood cultures (Bactec; Becton Dickinson, Sparks, MD), CBCs (Cell Dyne 4000; Abbott Diagnostics, Chicago IL), and UAs (Clinitek 200 Plus; Bayer Diagnostics, Elkhart, IN). High-sensitivity CRP was measured with the use of a BN Prospect nephelometer (Dade-Behring, Newark, DE), according to the manufacturer's instructions, with an analytical range of 0.175–1100 mg/L.

During January–December of 2001, we recruited 171 patients with suspected upper respiratory or other types of infections, and 90 healthy individuals with no apparent clinical symptoms of infection. Both inpatients and ambulatory patients who visited our clinics and agreed to participate in the study (during the time that the study coordinators were available) were enrolled in a consecutive manner. To enroll in the study, the patients signed an informed consent form (human studies approval number 631-00) approved by our institution's human studies review board. One random urine sample, one serum sample, one EDTA plasma sample, and one blood culture tube were obtained from each participant. The subjects' medical records were reviewed for admitting and discharge diagnoses. CBCs, blood cultures, and UAs were performed on the same day of sample collection. The urine and serum samples were stored at −70 °C until CRP, uTi, and creatinine concentrations were analyzed. Two reviewers (a board-certified microbiologist/pathologist and a clinical chemist) were blinded to the CRP and uTi results before they assigned the patients to one of four diagnostic categories, as described below. The reviewers had access to the patients' medical records and the UA and CBC results, including the percentages of various cells, to determine the diagnostic category for each patient. Increased granulocytes or the presence of bands were considered to indicate bacterial infection, and an elevated lymphocyte count was consistent with viral infection. Varying magnitudes between these two categories were used to assign the remaining patients to

the probable bacterial or probable viral infection groups. The four diagnostic categories are described as follows:

1. Bacterial: Blood culture is positive, WBCs and granulocytes are increased, and urine is nitrate-positive or has bacteria and WBCs.
2. Probable bacteria: WBCs and/or granulocytes are slightly elevated, and UA indicates bacterial infection but is equivocal.
3. Viral: Increased lymphocytes (percentage of lymphocytes is greater than that of granulocytes) and/or clinical impression based on medical records.
4. Probable viral: Lymphocytes are elevated, but their percentage is still less than that of granulocytes.

Patients with HIV and/or AIDS, leukemia, gross immunosuppression, or fungal or parasitic infections were excluded from the data set because of an expected muted or altered white cell response. We also excluded individuals from whom both urine and blood samples had not been collected. EP Evaluator Release 5 (David G. Rhoads Associates, Inc., Kennett Square, PA) was used for receiver operating characteristic (ROC) analysis. We performed a Bonferroni/Dunn post hoc test using Statview 4 Software (SAS Institute, Inc., Cary, NC) to compare the means of CRP and uTi in the control group to those in the four test groups.

RESULTS

The mean age for enrolled controls (n = 90) was 39.6 ± 11.6 years, which was not statistically different from that of the patients (n = 171; 41.1 ± 16.1 years; $P = 0.39$). Enrolled patients (n = 149) were grouped into the four categories of bacterial (n = 70), probable bacterial (n = 54), viral (n = 7), and probable viral groups (n = 18) according to the criteria outlined in the Materials and Methods section. A flow chart depicting the number of patients in each category is included in Fig. 1. The mean $\pm$ SE for absolute granulocyte and lymphocyte counts for all groups is also listed in Table 2. As expected, the highest counts for granulocytes and lymphocytes were found in the bacterial and viral groups, respectively. Both groups classified as probable had cell count means between those of the bacterial and viral groups, and the controls were within the reference range as expected.

The uTi concentrations in the controls and patients grouped into the four diagnostic categories are shown in Table 3. Normalization of the uTi values to urinary creatinine in the enrolled subjects did not significantly alter the results. Therefore, in this study, direct measurements of uTi in urine were compared with CRP values in serum.

The uTi and CRP concentrations measured in the controls and the four diagnostic groups are shown in Table 4. Both markers were significantly different in the bacterial vs. the probable bacterial group as assessed by Bonferroni/Dunn post hoc test ($P < 0.0001$). CRP was significantly different ($P < 0.0003$) in the bacterial group compared to the viral, probable viral, and probable bacterial groups. We also found that the uTi was significantly different in the bacterial group compared to the probable bacterial and probable viral groups ($P < 0.05$). (There were only seven subjects in the viral

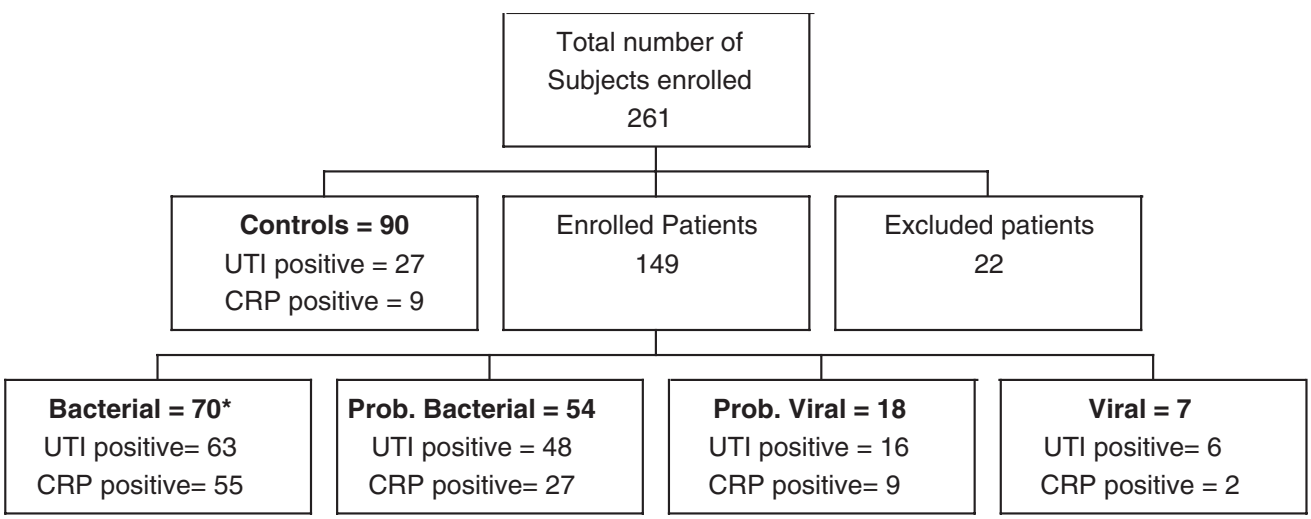

Fig. 1. Flow diagram of patients enrolled in the study. The excluded patients include those with hematologic malignancies or AIDS, or lacking a complete set of submitted urine and blood samples (see Materials and Methods section). *In the bacterial group, CRP was measured in 67 patients. Based on ROC analysis, the best cutoff values for uTi and CRP were 2.7 mg/L and 10.0 mg/L, respectively. In each diagnostic group, the numbers of patients with values above these cutoffs are indicated for each analyte.

TABLE 2. Concentrations (Mean ± SE) of uTi and CRP and cell counts in controls and the four diagnostic groups*

Group	uTi (mg/L)	CRP (mg/L)	WBC (4500–10,800)	Abs. gran (1500–7000)	Abs. lymph (1500–4000)
Controls	2.8 ± 0.8	2.7 ± 0.4	6173 ± 186	3376 ± 152	1891 ± 74
Bacterial	25.3 ± 3.1	99.0 ± 11.6	14,824 ± 1077	10,699 ± 778	1547 ± 128
Probable bacterial	16.1 ± 1.8	33.4 ± 6.7	8215 ± 315	5555 ± 255	1833 ± 98
Viral	16.9 ± 6.3	9.7 ± 5.7	6842 ± 942	2448 ± 578	3784 ± 488
Probable viral	15.1 ± 2.1	19.2 ± 5.9	6820 ± 657	3396 ± 462	1893 ± 282

*The reference ranges for cell counts are included in parenthesis.

TABLE 3. Creatinine normalization of uTi measurements in urine*

Group	N_{total}	uTi (mg/L) ± SE	N_{creat}	uTi /creatinine (mg/g) ± SE	*P*-value
Controls	90	2.8 ± 0.8	87	2.1 ± 0.6	0.70
Bacterial	70	25.3 ± 3.1	52	25.2 ± 4.2	0.90
Probable bacterial	54	16.1 ± 1.8	24	11.7 ± 3.2	0.71
Viral	7	16.9 ± 6.3	5	13.4 ± 2.8	0.37
Probable viral	18	15.1 ± 2.1	9	10.8 ± 2.3	0.15

*Means for each group were not different when normalized to creatinine ($P > 0.05$). N_{total} indicates the number of individuals in whom uTi was analyzed. N_{creat} indicates the number of individuals for whom urinary creatinine was also performed.

TABLE 4. Summary statistics for ROC curves depicted in Fig. 2

ROC curve	AUC ± SE	95% CI	Cutoff	Specificity	Sensitivity
Bacterial group					
CRP	0.921 ± 0.025	0.87–0.97	10.0 mg/L	95.6	82.1
uTi	0.889 ± 0.027	0.84–0.94	2.8 mg/L	82.2	91.0
Probable bacterial and bacterial groups					
CRP	0.874 ± 0.024	0.83–0.92	8.7 mg/L	94.4	70.2
uTi	0.884 ± 0.024	0.83–0.92	2.7 mg/L	82.2	90.1
Probable viral and viral groups					
CRP	0.782 ± 0.053	0.68–0.88	14.6 mg/L	97.8	44.0
uTi	0.880 ± 0.038	0.81–0.96	5.1 mg/L	85.8	88.0
All diagnostics groups					
CRP	0.858 ± 0.024	0.81–0.90	1.7 mg/L	61.1	89.0
uTi	0.884 ± 0.024	0.84–0.93	2.0 mg/L	80.0	91.1

group, and when their uTi concentration mean was compared to that of the bacterial group, it was not significantly different [$P = 0.15$]).

The ROC plots for CRP and uTi in the diagnostic groups, as outlined in the Materials and Methods section, are shown in Fig. 2. The corresponding ROC curve statistics are listed in Table 4. Since the number of subjects included in the viral group was small, no diagnostic comparisons were made for the two biomarkers in terms of diagnosing viral infection. However, the combined viral and probable viral groups were compared with the controls. uTi showed greater sensitivity for detecting a probable bacterial or viral case compared to CRP (Table 4). For example, for diagnosing infection in patients with suspected viral disease, uTi and CRP had sensitivities of 88.0% and 44.0%, respectively. The negative predictive value for uTi in diagnosing bacterial or probable bacterial patients was 86.0%, compared to 70.2% for CRP. As expected, CRP was a more specific marker for diagnosing bacterial infection, and the

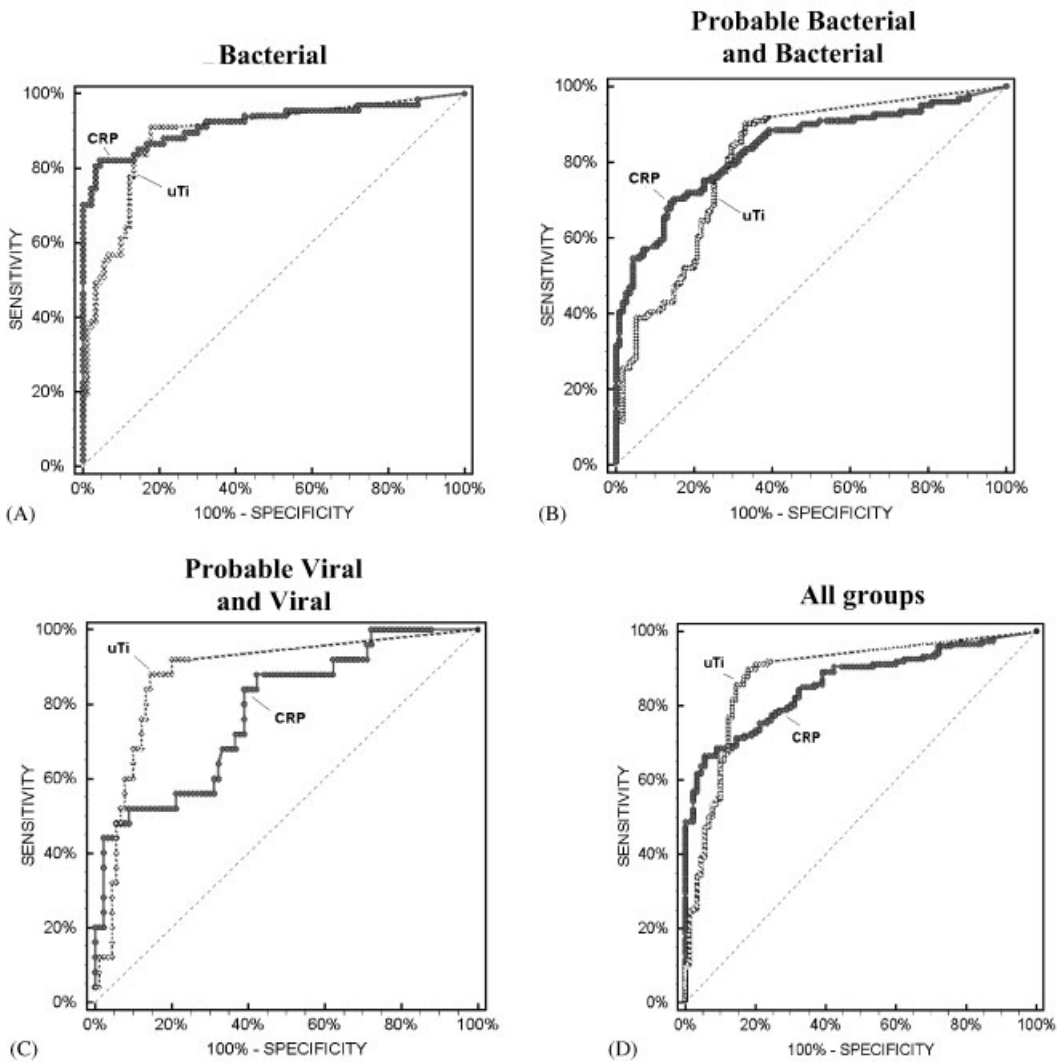

Fig. 2. ROC curves for Uristatin measured in the urine, and CRP measured in the serum of controls and patients diagnosed with bacterial (**A**), probable bacterial or bacterial (**B**), probable viral or viral (**C**), and all four diagnostic groups (**D**). The AUC and other summary statistics are listed in Table 4.

corresponding positive predictive values for uTi and CRP were 94.4% and 87%, respectively.

DISCUSSION

In this study we assessed the utility of uTi measured in urine for diagnosing infection in comparison with serum CRP and clinical end-points such as white cell response to inflammation.

Nearly a century ago, it was reported that urine collected from patients with various infections (such as pneumonia or intestinal typhus), kidney disease, or toxemia showed increased trypsin inhibitory activity (15,16). uTi in urine was first isolated in pregnant women in 1955 (17). Further work revealed that uTi is a Kunitz-type protease with inhibitory effects on enzymes such as trypsin, chymotrypsin, esterases, and hyaluronidases (18). Since uTi inhibits uterine contractility in pregnancy, it has been evaluated and used clinically for the treatment of premature labor (19,20). Intraperitoneal administration of uTi to rats prior to an injection of staphylococcal enterotoxin B has been shown to reduce lung injury caused by the toxin (21). However, the mechanism behind the defensive effect of uTi has yet to be determined.

Merle et al. (9) reported that uTi concentrations are independent of serum creatinine. Our results show that normalization with urine creatinine did not change the

relative means of uTi in the urine of controls and patient groups (Table 2). Therefore, it apparently is possible to measure the concentration of uTi in the urine to diagnose infection or inflammation.

As regards the diagnosis of bacterial infection, previous studies have demonstrated that concentrations of trypsin inhibitors in the urine increase in cases of bacterial infection (7,9,10). Each of these studies assessed the utility of this biomarker in a select group of patients. For example, in the study by Merle et al. (9), individuals over the age of 60 years were included, and a more recent study by Pugia et al. (7) enrolled Japanese school children as subjects. In a large prospective study of patients in internal-medicine clinics, Piette et al. (10) reported that patients who had been diagnosed with bacterial or viral infections or cancer had higher concentrations of uTi compared to controls (7).

In the current study, uTi showed a greater clinical sensitivity than CRP for diagnosing infection. Our data also show that CRP is a more specific marker for diagnosing bacterial infection. These results are consistent with those of Merle et al. (9), who concluded that uTi increased significantly in the urine of elderly people with bacterial infections. In their study, CRP also had a greater specificity for diagnosing bacterial infection.

As shown in Table 4, the overall area under the curve (AUC) for both uTi and CRP ranged from 0.782 to 0.921 in all of the diagnostic groups. The AUC values for uTi and CRP in the bacterial group were not significantly different ($P = 0.37$), nor were they different for all four groups combined ($P = 0.42$). The positive and negative predictive values of the uTi assay at a cutoff of 2.8 mg/L for diagnosing bacterial infection were 79% and 93%, respectively. For detecting inflammation (as indicated by white cell response), uTi had a positive predictive value of 73% and a negative predictive value of 88%. At the same cutoff value, uTi also performed well for ruling out infection as assessed by increased white cell response.

Considering the fact that both CRP and uTi exhibited an adequate performance in diagnosing bacterial infection, it is noteworthy that uTi was performed in a random urine sample, whereas CRP was measured in serum. The ability of a urinary marker to perform as well as a serum marker in diagnosing bacterial infection provides an opportunity for developing a noninvasive marker for rapid screening of patients in a variety of settings, such as at bedside or in physicians' offices. Pugia et al. (7) recently reported that a dipstick reading on a reflectance photometer to measure uTi in urine had an 85% concordance with an immunoassay developed for this analyte to diagnose inflammation. Although CRP is a more specific marker for diagnosing bacterial infection, it remains a nonspecific marker of infection. The greater sensitivity of uTi (90.1%) vs. that of CRP (70.2%) suggests that the uTi test may be more suitable for assessing infection, particularly because it is noninvasive. Further studies are needed to assess the utility of this marker in a point-of-care format for managing patients with suspected infections or inflammation.

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