# Evaluation of Novel Urinary Tract Infection Biomarkers in Children

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## Abstract

**Background**—Pediatricians frequently use urinalysis to diagnose urinary tract infection (UTI) while awaiting urine culture results, but sensitivity and specificity of urinalysis are limited. This study evaluated the diagnostic accuracy of the antimicrobial peptides human α-defensin 5 (HD5) and human neutrophil peptides (HNP) 1-3 as novel UTI biomarkers in children.

**Methods**—We prospectively enrolled 199 pediatric Emergency Department or Urgent Care patients evaluated for a UTI. Urine concentrations of HD5 and HNP1-3 were measured by enzyme-linked immunosorbent assay. Urine culture was the reference standard. Sensitivities and specificities of leukocyte esterase (LE), HD5, HNP1-3, and test combinations were compared.

**Results**—For predicting positive urine culture, the areas under the receiver operating characteristic curves for HD5 and HNP1-3 were 0.86 (95% CI, 0.81-0.92) and 0.88 (95% CI, 0.82-0.93), respectively. Compared to LE   trace, the combination test "LE and HD5" increased specificity by 6% (95% CI, 3%-10%) without decreasing sensitivity. In the subgroup whose urine was collected by a clean-catch method, combination tests "LE and HD5" and "HD5 and HNP1-3" increased specificity by > 10% compared to LE alone.

**Conclusion**—Urine antimicrobial peptide profiles are a promising novel strategy as an adjunct to urinalysis to aid UTI diagnosis in children.

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## Introduction

Urinary tract infection (UTI) is a common and potentially severe infection that represents a major burden of healthcare utilization/expenditure and antibiotic exposure in children (1-3). The diagnosis of UTI relies on suggestive symptoms, pyuria, and isolation of a uropathogen in culture. However, UTI symptoms in children often are non-specific (4), and culture results are not available at the time of initial evaluation. Consequently, providers frequently rely on rapid results of the urine dipstick to decide whether to initiate empiric antibiotic therapy for a presumed UTI while awaiting culture results. This approach may lead to either delayed diagnosis or unnecessary antibiotic therapy because of the limitations of leukocyte esterase (LE) and urinary nitrite on dipstick urinalysis. Specifically, large pediatric studies and meta-analyses of the utility of the urine dipstick for predicting positive urine culture have consistently shown suboptimal sensitivity and/or specificity of individual and combination tests, even though results of individual studies vary widely (5-8).

In this study, we evaluated two peptides in the α-defensin family of antimicrobial peptides (AMPs) as novel biomarkers for UTI in children. AMPs are small, cationic peptides that participate in the innate immune defense of the kidneys, urinary tract, and other organ systems (9). AMPs are produced by white blood cells and/or epithelial cells and may be constitutively expressed or induced when pathogens enter the urinary tract (9). Human α-defensin 5 (HD5) is an epithelial-derived AMP produced by intestinal Paneth cells, the female genital tract, and the uroepithelium (10-12). In a prior study of a small number of children, our research group demonstrated via Western immunoblot and enzyme-linked immunosorbent assay that HD5 was detected in culture-positive but not culture-negative urine samples (12). Human neutrophil peptides (HNP) 1-3 are three closely related α-defensins (collectively known as HNP1-3 and typically measured in aggregate) produced in promyelocytes and stored in the primary granules of neutrophils (9). Two prior studies of adults demonstrated increased urine concentrations of HNP1-3 during acute UTI (13) and HNP1 during chronic pyelonephritis (14). Given this prior data, we hypothesized that HD5 and HNP1-3, alone or as adjuncts to dipstick urinalysis, would improve diagnostic accuracy for UTI. Therefore, the objective of this study was to evaluate the performance of urine HD5 and HNP1-3 concentrations as biomarkers for positive urine culture in children.

## Results

### Study population

During the study period, urine samples were collected from 268 Emergency Department (ED) or Urgent Care (UC) patients who met inclusion criteria. Of those, 34 had received antibiotics within 7 days before presentation and were excluded. Of the remaining 234 patients, 199 provided adequate excess urine sample volume for analysis of both HD5 and HNP1-3 concentrations. Table 1 describes the demographic characteristics and documented clinical signs and symptoms of the study population.

### Clinical laboratory results

Of the 199 patients, 29 (15%) had urine cultures that yielded  50 000 CFU/mL of a single uropathogen. The positive urine cultures included 10 (10%) of 99 in the subgroup of patients whose urine was collected by catheterization and 19 (19%) of 100 in the subgroup whose urine was collected by a clean-catch method. *Escherichia coli* was the most common bacterial isolate, accounting for 25 (86%) positive urine cultures, followed by *Staphylococcus saprophyticus* (n = 2), *Klebsiella pneumoniae* (n = 1), and *Serratia marcescens* (n = 1). Of the 29 patients with a positive urine culture, 23 (79%) had signs or symptoms of upper urinary tract infection, including fever, back/flank pain, costovertebral angle tenderness, or vomiting. Table 2 lists the sensitivities and specificities of LE and nitrite, alone and in combination, for all patients and for subgroups by urine collection method.

### Antimicrobial peptide analyses

Urine HD5 and HNP1-3 concentrations were significantly higher in culture-positive urine samples compared to culture-negative urine samples (Figure 1, Panels A and B). The median HD5 concentration was 590.6 pg per mg creatinine (pg/mgCr) (IQR, 373.1-908.5) in culture-positive urine samples versus 89.6 pg/mgCr (IQR, 12.8-253.5) in culture-negative urine samples ($P < .001$, Mann-Whitney). The median HNP1-3 concentration was 3801.4 pg/mgCr (IQR, 1776.6-11 861.3) in culture-positive urine samples versus 148.7 pg/mgCr (IQR, 25.7-934.2) in culture-negative urine samples ($P < .001$, Mann-Whitney). The areas under the ROC curves for HD5 and HNP1-3 were 0.86 (95% CI, 0.81-0.92) and 0.88 (95% CI, 0.82-0.93), respectively (Figure 1, Panels C and D). Sensitivities and specificities of multiple test thresholds across the range of HD5 and HNP1-3 values are displayed in Supplemental Tables S1 and S2 (online).

### Test comparisons

The sensitivity of the test "LE  trace" was 97%. HD5 and HNP1-3 thresholds of 174 pg/mgCr and 384 pg/mgCr, respectively, provided maximal specificity of each individual test with sensitivity at least equal to that of "LE  trace". Comparisons of the sensitivities and specificities of LE, HD5, and HNP1-3, alone and in combination, for all patients are shown in Figure 2, Panel A. Nitrite was not included in these test comparisons due to its low sensitivity. The specificities of both HD5 and HNP1-3 were lower than the specificity of LE. However, the combination tests "LE and HD5" and "LE and HD5 and HNP1-3" increased specificity by 6% (95% CI, 3%-10%) and 7% (95% CI, 3%-11%), respectively, compared to LE alone without decreasing sensitivity.

### Analyses by urine collection method

Comparisons of LE, HD5, and HNP1-3 in the catheterization subgroup showed no increased specificity of combination tests versus LE alone (Figure 2, Panel B). However, in the clean-catch subgroup, specificity increased 13% (95% CI, 6%-21%) when "LE and HD5" was compared to LE alone (Figure 2, Panel C). The combination tests "HD5 and HNP1-3" and "LE and HD5 and HNP1-3" also increased specificity but differed only slightly from "LE and HD5".

## Discussion

In this prospective study, we evaluated two AMPs as novel biomarkers for pediatric urinary tract infection. We demonstrated that both HD5 and HNP1-3 performed well as diagnostic tests to predict positive urine culture in children, with areas under the ROC curves of 0.86 and 0.88 for HD5 and HNP1-3, respectively. For test comparisons, we sought to determine whether combinations of highly sensitive tests would increase specificity without decreasing sensitivity. When compared to LE alone, the combination test "LE and HD5" increased specificity by 6% in all patients and 13% in the clean-catch subgroup without affecting sensitivity. The addition of HNP1-3 to LE did not improve specificity, but the combination test "HD5 and HNP1-3" did increase specificity by 12% in the clean-catch subgroup. The combination test "LE and HD5 and HNP1-3" also increased specificity in all patients and the clean-catch subgroup, but the results were only slightly better than "LE and HD5" without HNP1-3. These results suggest that urine AMPs have potential to improve standard methods for diagnosing UTI in children.

The rationale for identifying novel UTI biomarkers stems from the limited utility of currently available point-of-care tests for the diagnosis of UTI in children. The high specificity (99%) but low sensitivity (45%) of urinary nitrite in our cohort is comparable to results of prior studies (5-8). Thus, clinicians can expect a positive urine culture when nitrite is detected, but a negative nitrite test result does not rule out a UTI. Regarding LE, three pediatric meta-analyses and one recent, large study reported sensitivities of 72-83% and specificities of 78-87% (5-8). In our study, LE sensitivity (97%) was greater than results of prior studies, while specificity (75%) was comparable. A high false-positive rate of LE may lead to over-diagnoses and unnecessary antibiotic exposure. False-positive LE tests occur because pyuria is associated with a number of other conditions including acute febrile illnesses, urinary calculi, sexually transmitted infections, intrinsic renal diseases, and other disorders (15). Congruent results of LE and nitrite (both positive or both negative) help to rule in or rule out a UTI (16,17), but the common scenario in which LE is positive but nitrite is negative creates diagnostic uncertainty. Improved rapid tests are needed to aid the accurate diagnosis of UTI.

In our study, both HD5 and HNP1-3 demonstrated potential to improve UTI diagnostic accuracy. Our results show that HD5 and HNP1-3 are induced during UTI. The higher concentrations of HD5 and HNP1-3 in culture-positive than culture-negative urine samples in our study is consistent with prior investigations performed in small numbers of children and adults (12,13). The areas under the ROC curves for HD5 and HNP1-3 were between 0.75 and 0.9 and thus generally indicate "good" overall diagnostic value of the biomarkers (18). However, HNP1-3 did not improve specificity when combined with LE, perhaps because LE and HNP1-3 are both neutrophil markers and therefore indicate pyuria. In contrast, HD5 is expressed throughout the urothelium of the lower urinary tract and in the nephron and collecting tubules of the kidney (12). As an epithelial-derived AMP, HD5 likely performed well as a UTI biomarker independent of pyuria. Indeed, we found that the addition of HD5 to LE improved specificity. Still, there were some false-positive HD5 test results. We speculate that reasons for false-positive HD5 tests may include urethritis, viral cystitis, UTI due to pathogens that do not grow well in culture, non-infectious inflammatory

conditions of the kidneys and urinary tract, and possibly other currently unrecognized stimuli for HD5 expression in the urine. Additionally, contamination of urine specimens with vaginal secretions could potentially result in false-positive HD5 test results because HD5 is produced in the female genital tract (11).

Novel UTI biomarkers may provide more benefit in certain clinical scenarios than others. In our study, the specificity of LE was much lower in the clean-catch subgroup. The data does not differentiate whether the difference in specificity was, in full or in part, due to the method of collection. Age, toilet-training status, or combinations of factors may have accounted for the difference. We are not aware of previous studies that directly compared LE specificity in samples collected by catheterization versus a clean-catch method. A previous study did compare characteristics of the urine dipstick by age group and found lower specificity of the combination test "LE or nitrite" in children aged    2 years (of whom only 10% were catheterized) than children aged < 2 years (of whom 88% were catheterized) (19). Whatever the reasons for the difference, in our study the greatest improvement in specificity was observed in the clean-catch subgroup, in whom combination tests "LE and HD5", "HD5 and HNP1-3", and "LE and HD5 and HNP1-3" all increased the specificity by > 10% compared to LE alone. Clinically, fewer false-positive tests may translate to decreased unnecessary empiric antibiotic therapy for patients evaluated for a UTI.

Our study has some limitations. First, we included all patients in whom urine testing was performed at the discretion of the treating clinician, irrespective of the pre-test probability for UTI. Although the majority of the study population had at least 1 sign or symptom potentially suggestive of a UTI, perhaps test characteristics would differ in a cohort limited to only those patients with a high pre-test probability (20). Next, two urinalysis machines were used in our study, depending on whether the dipstick analysis was performed in the ED/UC laboratories or the hospital laboratory. Dipstick results may differ at other centers that use different urinalysis machines. Last, the definition for a positive urine culture has not been established for all age groups and methods of urine collection. We chose a single definition for all patients based on current studies and guidelines (6,21-23).

In summary, we found that urine HD5 and HNP1-3 concentrations performed well as biomarkers for predicting positive urine culture in children. When combined with LE, HD5 provided the greatest increase in specificity without decreasing sensitivity, particularly when a clean-catch method of urine collection was used. We conclude that urine AMP profiles are a promising novel strategy as an adjunct to dipstick urinalysis to aid diagnosis and guide empiric management when UTI is suspected. Future research is needed to evaluate additional AMPs that may provide further improvements in specificity and/or sensitivity. Also, determination of AMP performance in subgroups of patients, such as those with neurogenic bladder and complicated urinary tracts, warrants investigation. Finally, development of rapid assays for urine AMP concentrations will ultimately be necessary to evaluate them as novel biomarkers at the point of care.

## Methods

### Study enrollment

Children were prospectively enrolled in the ED and main campus UC at Nationwide
Children's Hospital, Columbus, OH, from 11/20/2013 to 7/2/2014. Patients aged    18 years
who met the following inclusion criteria were enrolled during times when research staff was
available: 1) both dipstick urinalysis and urine culture were performed for any clinical
indication, and 2) excess urine sample was available. Patients were excluded if 1) they had
received antibiotics in the 7 days before presentation to the ED or UC, or 2) the urine sample
collected had insufficient volume for performance of all investigational assays. The
institutional review board at Nationwide Children's Hospital approved the study and granted
a waiver of informed consent (IRB13-00090).

### Clinical data collection and analysis

Electronic medical records were reviewed for pertinent demographic, clinical, and
laboratory data, including urine dipstick and urine culture results. Fever was considered
present when reported by the patient or caregiver or when a temperature    38°C was
documented in the ED/UC. Dipstick urinalyses were performed either in the ED or UC
laboratory using the CLINITEK Status®+ Analyzer (Siemens, Tarrytown, NY, USA) or in
the hospital chemistry laboratory using the iChem® Velocity™ instrument (Iris Diagnostics,
Chatsworth, CA, USA). Urine was plated by clinical microbiology laboratory staff using
calibrated loops and cultured on 5% sheep blood and MacConkey agar biplates and
incubated at 35˚C in ambient atmosphere. A positive urine culture result consisted of    50
000 colony forming units (CFU) per mL of a single uropathogen (21,23). Urine cultures
were considered negative if they yielded < 50 000 CFU/mL, mixed bacteria, or likely
contaminants such as *Lactobacillus* species, coagulase-negative staphylococci, or
*Corynebacterium* species (23).

### Sample collection

After ensuring sufficient urine volume was available for clinical diagnostic tests, excess
urine was immediately collected in AssayAssure™ urine collection tubes (Thermo
Scientific™, Waltham, MA, USA) containing a bacteriostatic preservative that suppresses
nuclease and protease activity and preserves urine specimens at room temperature for up to
26 days according to the manufacturer.

### AssayAssure™ validation

We independently verified the stability of AMPs for up to 14 days in AssayAssure™
preservative by measuring serial urine concentrations of ribonuclease 7 (RNase 7). We chose
to measure RNase 7 because it is an AMP that is constitutively expressed in the urine of
healthy individuals. On day 0, urine samples from 2 healthy individuals were collected and
stored in AssayAssure™ urine collection tubes. On days 1, 2, 7, and 14, an aliquot of each
sample was removed and stored at -80°C until analyzed. After the 14 day period, all frozen
samples were analyzed simultaneously in a single enzyme-linked immunosorbent assay to
determine the concentrations of RNase 7, as previously described (24). Concentrations of

RNase 7 remained stable in AssayAssure™ during the 14 day validation period
(Supplemental Figure S1 (online)).

## Sample processing and analysis

Within 7 days of collection, study samples were centrifuged at 3000 rpm for 5 minutes. The
supernatant was saved in 300 to 500 μL aliquots and stored at -80°C until analyzed. To
evaluate subgroups by urine collection method, nearly equal numbers of study samples
obtained by catheterization and clean-catch method were analyzed. Urine concentrations of
HD5 and HNP1-3 were measured in duplicate by enzyme-linked immunosorbent assay
using commercial kits according to the manufacturers' instructions (HD5: Uscn Life Science
Inc., Wuhan, Hubei, China; HNP13: Hycult Biotech Inc., Plymouth Meeting, PA, USA).
Study samples with concentrations less than the lower limit of quantification for HD5 or
HNP1-3 were assigned a value of one-half the lower limit of quantification as calculated on
a $\log_{10}$ curve (25). To account for urine dilution, concentrations of HD5 and HNP13 were
normalized to urine creatinine concentration, which was measured by a colorimetric assay
(Oxford Biomedical Research, Oxford, MI, USA).

## Sample size determination

To design a study with 80% power, 176 total patients was adequate to ascertain a 10%
difference in specificity between two diagnostic tests with a Type I error of 0.05 (26).

## Statistical analysis

Urine culture was the reference standard for evaluating test characteristics of the urine
dipstick and AMPs. First, urine concentrations of each AMP were compared in culture-
negative versus culture-positive urine samples using the Mann-Whitney rank-sum test (non-
normally distributed, continuous variables). Next, a receiver operating characteristic curve
was generated for each AMP, and the area under the curve was calculated using the scientific
software GraphPad Prism 6 (GraphPad Software Inc, La Jolla, CA, USA). Optimal threshold
values for positive HD5 and HNP1-3 test results were modeled to maximize specificity
while ensuring sensitivity no less than that of LE. Last, the sensitivities and specificities of
LE, HD5, HNP1-3, and combinations of the aforementioned tests were compared. Test
combinations using the conjunction "and" were considered positive only if all components
were positive. Test combinations using the conjunction "or" were considered positive if any
individual component was positive. Comparisons between subgroups utilized Fisher's exact
test (categorical variables) or Mann-Whitney rank-sum test (non-normally distributed,
continuous variables). McNemar's test was used to evaluate differences in specificity
between two diagnostic tests. All *P* values were two-sided.

## Acknowledgments

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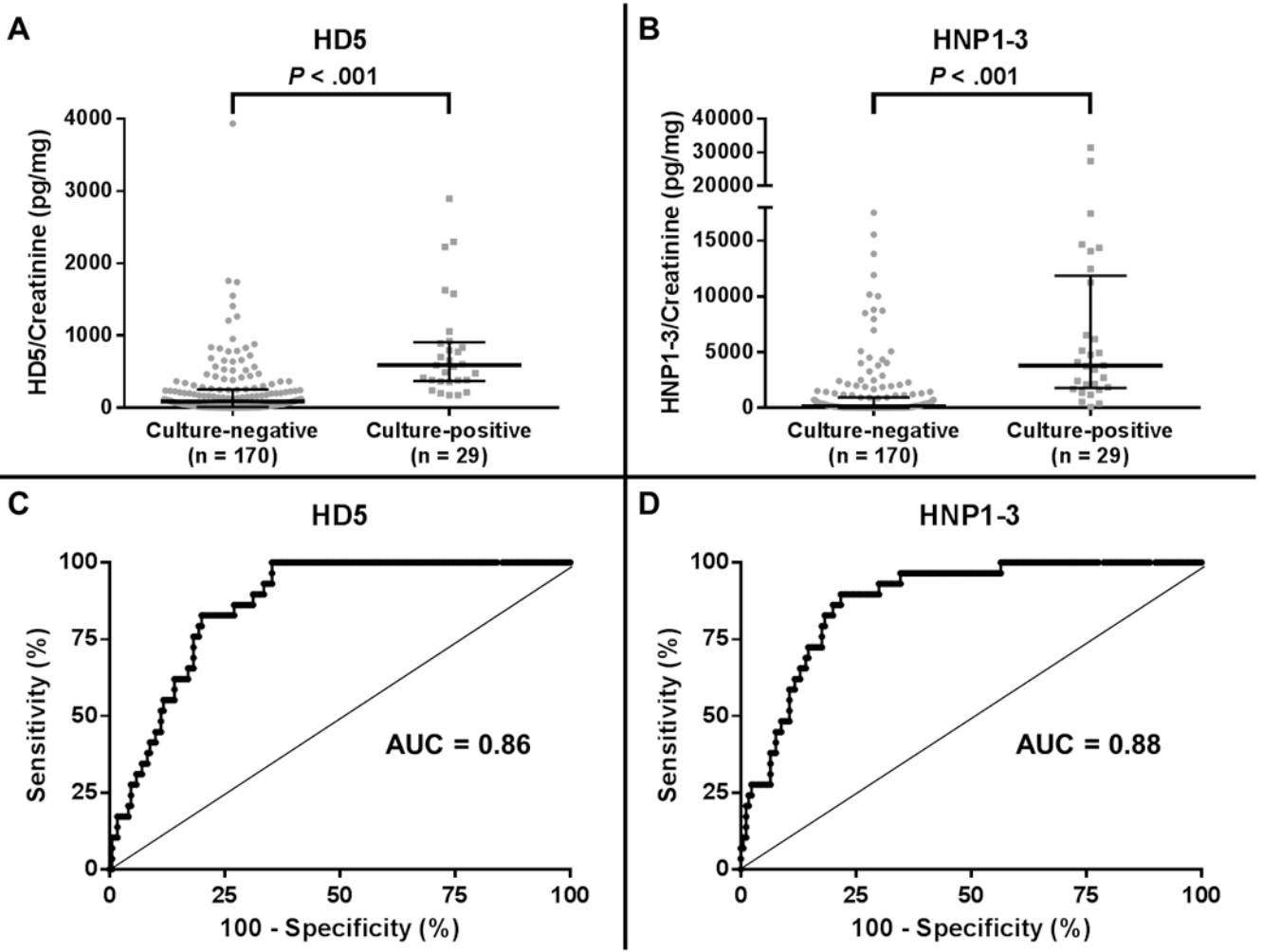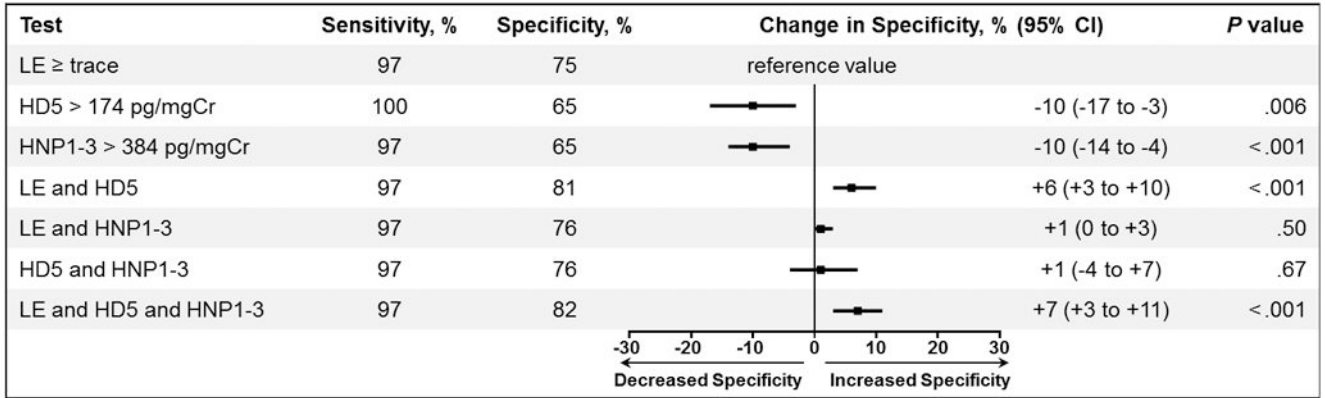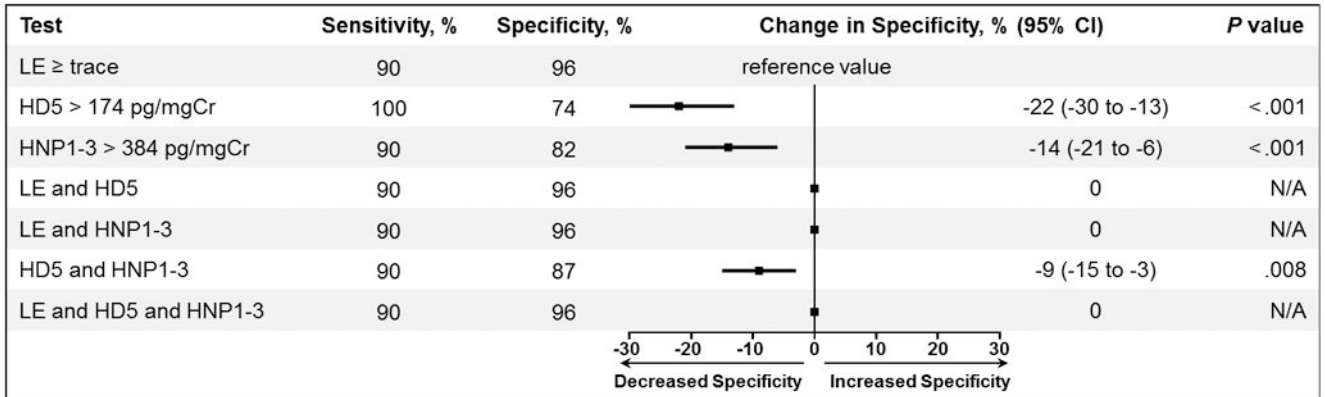
**Figure 1. HD5 and HNP1-3 concentrations in culture-negative and culture-positive urine samples (A/B), and ROC curves (C/D)**

A/B, Horizontal bars represent median values and interquartile ranges. Gray circles and squares indicate individual data points. C/D, The diagonal line represents a test with no diagnostic value.

## A

| Test | Sensitivity, % | Specificity, % | Change in Specificity, % (95% CI) | | P value |
|---|---|---|---|---|---|
| LE ≥ trace | 97 | 75 | reference value | | |
| HD5 > 174 pg/mgCr | 100 | 65 | | -10 (-17 to -3) | .006 |
| HNP1-3 > 384 pg/mgCr | 97 | 65 | | -10 (-14 to -4) | <.001 |
| LE and HD5 | 97 | 81 | | +6 (+3 to +10) | <.001 |
| LE and HNP1-3 | 97 | 76 | | +1 (0 to +3) | .50 |
| HD5 and HNP1-3 | 97 | 76 | | +1 (-4 to +7) | .67 |
| LE and HD5 and HNP1-3 | 97 | 82 | | +7 (+3 to +11) | <.001 |

-30  -20  -10  0  10  20  30
Decreased Specificity    Increased Specificity

## B

| Test | Sensitivity, % | Specificity, % | Change in Specificity, % (95% CI) | | P value |
|---|---|---|---|---|---|
| LE ≥ trace | 90 | 96 | reference value | | |
| HD5 > 174 pg/mgCr | 100 | 74 | | -22 (-30 to -13) | <.001 |
| HNP1-3 > 384 pg/mgCr | 90 | 82 | | -14 (-21 to -6) | <.001 |
| LE and HD5 | 90 | 96 | | 0 | N/A |
| LE and HNP1-3 | 90 | 96 | | 0 | N/A |
| HD5 and HNP1-3 | 90 | 87 | | -9 (-15 to -3) | .008 |
| LE and HD5 and HNP1-3 | 90 | 96 | | 0 | N/A |

-30  -20  -10  0  10  20  30
Decreased Specificity    Increased Specificity

## C

| Test | Sensitivity, % | Specificity, % | Change in Specificity, % (95% CI) | | P value |
|---|---|---|---|---|---|
| LE ≥ trace | 100 | 52 | reference value | | |
| HD5 > 174 pg/mgCr | 100 | 54 | | +2 (-8 to +13) | .65 |
| HNP1-3 > 384 pg/mgCr | 100 | 47 | | -5 (-12 to +2) | .29 |
| LE and HD5 | 100 | 65 | | +13 (+6 to +21) | <.001 |
| LE and HNP1-3 | 100 | 54 | | +2 (-1 to +6) | .50 |
| HD5 and HNP1-3 | 100 | 64 | | +12 (+4 to +21) | .01 |
| LE and HD5 and HNP1-3 | 100 | 67 | | +15 (+7 to +23) | <.001 |

-30  -20  -10  0  10  20  30
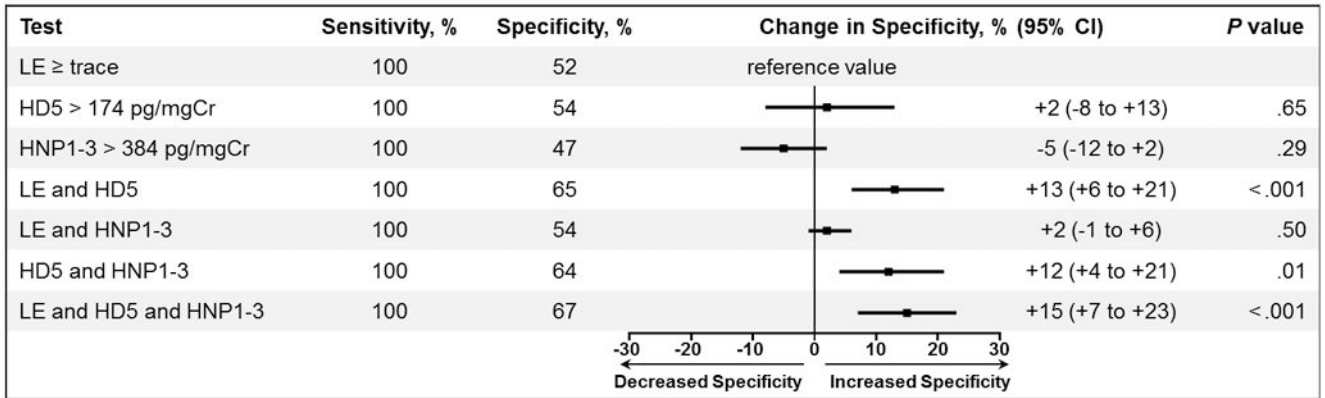Decreased Specificity    Increased Specificity

**Figure 2. Comparisons of sensitivity and specificity of LE, HD5, and HNP1-3, alone and in combination**

A, All study patients. B, Catheterization subgroup. C, Clean-catch subgroup. Graphs display the change in specificity of each individual or combination test compared to LE trace. Horizontal bars represent 95% confidence intervals. N/A, not applicable.

**Table 1**

**Demographic Characteristics and Documented Clinical Signs and Symptoms of Study Population[a]**

| | All Patients (n = 199) | Catheterization[b] (n = 99) | Clean-catch[c] (n = 100) | Catheterization versus Clean-catch *P* value[d] |
|---|---|---|---|---|
| Age in years, median (IQR) | 5.0 (1.2-12.8) | 1.2 (0.4-3.9) | 10.0 (5.4-14.6) | < .001 |
| Female | 156 (78) | 69 (70) | 87 (87) | < .001 |
| Fever | 95 (48) | 67 (68) | 28 (28) | < .001 |
| Abdominal pain | 71 (36) | 18 (18) | 53 (53) | < .001 |
| Dysuria | 45 (23) | 10 (10) | 35 (35) | < .001 |
| Urinary frequency | 19 (10) | 4 (4) | 15 (15) | .01 |
| Urinary urgency or incontinence | 13 (7) | 0 | 13 (13) | < .001 |
| Back/flank pain or CVA tenderness | 24 (12) | 4 (4) | 20 (20) | < .001 |
| Vomiting | 59 (30) | 34 (34) | 25 (25) | .16 |
| 1 of above signs/symptoms | 177 (89) | 87 (88) | 90 (90) | .66 |

CVA, costovertebral angle

[a]Unless otherwise specified, numerical values indicate the number (percent) with the stated demographic characteristic or clinical sign or symptom.

[b]Subgroup of patients whose urine was collected by catheterization

[c]Subgroup of patients whose urine was collected by a clean-catch method

[d]Fisher's exact test (categorical variables) or Mann-Whitney rank-sum test (age)

**Sensitivities and Specificities of Leukocyte Esterase and Nitrite, Alone and in Combination**

| Test Threshold | All Patients (n = 199) | | Catheterization[a] (n = 99) | | Clean-catch[b] (n = 100) | |
|---|---|---|---|---|---|---|
| | Se, % | Sp, % | Se, % | Sp, % | Se, % | Sp, % |
| LE trace | 97 | 75 | 90 | 96 | 100 | 52 |
| LE small | 83 | 85 | 90 | 97 | 79 | 72 |
| LE moderate | 41 | 94 | 30 | 100 | 47 | 86 |
| LE = large | 28 | 98 | 20 | 100 | 32 | 96 |
| Nitrite | 45 | 99 | 40 | 99 | 47 | 99 |
| LE trace and nitrite | 45 | 99 | 40 | 99 | 47 | 99 |
| LE trace or nitrite | 97 | 75 | 90 | 96 | 100 | 52 |

LE, leukocyte esterase; Se, sensitivity; Sp, specificity

[a] Subgroup of patients whose urine was collected by catheterization

[b] Subgroup of patients whose urine was collected by a clean-catch method