A Real-Time PCR-Based Semi-Quantitative Breakpoint to Aid in Molecular Identification of Urinary Tract Infections

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Abstract

This study presents a novel approach to aid in diagnosis of urinary tract infections (UTIs). A real-time PCR assay was used to screen for culture-positive specimens and to identify the causative uropathogen. Semi-quantitative breakpoints were used to screen for significant bacteriuria (presence of \( \geq 10^2 \) CFU/ml of uropathogens) or low-level bacteriuria (containing between \( 10^3 \) and \( 10^4 \) CFU/ml of uropathogens). The 16S rDNA-based assay could identify the most prevalent uropathogens using probes for *Escherichia coli*, *Pseudomonas* species, *Pseudomonas aeruginosa*, *Staphylococcus* species, *Staphylococcus aureus*, *Enterococcus* species and *Streptococcus* species. 330 urinary specimens were analysed and results were compared with conventional urine culture. Using a PCR Ct value of 25 as semi-quantitative breakpoint for significant bacteriuria resulted in a sensitivity and specificity of 97% and 80%, respectively. In 78% of the samples with monomicrobial infections the assay contained probes to detect the bacteria present in the urine specimens and 99% of these uropathogens was correctly identified. Concluding, this proof-of-concept approach demonstrates that the assay can distinguish bacteriuria from no bacteriuria as well as detect the involved uropathogen within 4 hours after sampling, allowing adequate therapy decisions within the same day as well as drastically reduce consequent urine culturing.

Introduction

Molecular techniques are becoming an integral part of a diagnostic microbiological laboratory. Many microbiological laboratories performing real-time PCR already offer a broad panel of bacterial and viral targets. Though, for some infections conventional testing procedures are still being used. Urinary tract infections (UTIs) comprise one of the largest classes of infections occurring both in hospital and in community [1,2,3]. The diagnosis of UTIs is based on semi-quantitative urine culture, used as reference standard, which provides both quantification as well as identification of the uropathogen. Quantification of uropathogens is essential as different bacterial loads (>\( 10^3 \) or \( 10^5 \) CFU/ml) are used in combination with clinical symptoms to diagnose UTIs. The diagnosis might prove essential in rapid adequate treatment.

To increase the rapidity of identification of UTIs, rapid urinalysis tools are available and include for example testing for nitrite and leukocytes, and microscopic sediment analysis for bacteria and white blood cells. These screening tools are fast, but often lack sensitivity [8,9]. Reports associated the nitrite test with a sensitivity and specificity of 45–60% and 85–98%, respectively, leukocyte-esterase testing has been shown to have sensitivity and specificity rates of 48–86% and 17–93%, respectively [8]. The rapidity and ease of use of urine dipsticks are particularly preferred by general practitioners, whereas microbiological laboratories demand solutions designed for high-throughput analysis. An example of an automated urinalysis instrument is the Sysmex urine fluorescence flow cytometer (Sysmex UF-1000i). This instrument uses an algorithm which combines the quantitative detection of bacteria and white blood cells to determine if infection is present. Although the Sysmex system, in contrast to the dipsticks, is able to adjust settings to cover either bacterial loads \( \geq 10^3 \) or \( 10^5 \) CFU/ml, this system, as well as the dipstick testing, shows limitations in clinical sensitivity and does not provide an identification of the uropathogen involved. Especially in a hospital setting, where often a larger variation in UTI aetiology is seen as compared to at the general practitioners, uropathogen identification might prove essential in rapid adequate treatment.

The aim of this study was to develop a new molecular approach accounting for both rapid semi-quantification of the bacterial load in urine as well as the identification of the uropathogen. This UTI screening assay was based on the earlier described multi-probe 16S rDNA-based real-time PCR assay using species- and genus-specific probes [10]. The most promising new feature evaluated in the current study was the semi-quantitative breakpoint to distinguish between positive and negative UTI samples. The discriminatory breakpoint was based on the scattering in cycle threshold (Ct) values of the universal 16S rDNA probe. In this study, we defined significant bacteriuria as a uropathogen load...
≥10^3 CFU/ml. Bacterial loads below 10^3 CFU/ml were considered to be no UTI. In specific patient groups it could be indicated to include a subgroup of low-level bacteriuria as a load between 10^3–10^4 CFU/ml. The detection of this group was also investigated in the study. The other purpose of the UTI screening tool was to offer a rapid indication about the causative agent. The pathogens selected as target in the real-time PCR assay were *Escherichia coli*, *Pseudomonas* species, *Pseudomonas aeruginosa*, *Staphylococcus* species, *Staphylococcus aureus*, *Enterococcus* species, and *Streptococcus* species.

**Methods**

**Ethics Statement**

All data in this study were analyzed anonymously, and the samples were considered to be medical waste materials. Therefore, no consent from the patients was required and the ethics committee did not have to be approached. This is in agreement with the code for proper use of human tissue as formulated by the Dutch Federation of Medical Scientific Societies and the policy of the Medical Ethics Committee of the Maastricht University Medical Center.

**Clinical Samples**

We included 330 randomly selected routine clinical urine samples submitted to the Medical Microbiology Laboratory of the Maastricht University Medical Center (MUMC, the Netherlands) for urine culture analysis. The samples were collected from symptomatic and asymptomatic in- and outpatients. The median age of the patients was 64 years old (range 0–92 years, 10% <20 years, 21% 21–50 years, 69% >50 years old), 56% of the patients were female. Conventional processing of the urine specimens consisted of dipstick testing for nitrite and/or further pathogen identification by Gram-staining and biochemical testing. Samples were cultured using standard microbiological methods. Identification and antibiotic susceptibility testing was performed with the Phoenix system (BD). All samples were used according to the code for proper use of human tissue as formulated by the Dutch Federation of Medical Scientific Societies.

**Processing of Urine Samples**

1 ml of urine sample was centrifuged for 5 min (13,400×g). The supernatant was removed and the remaining pellet was washed with 900 μl phosphate buffered saline (PBS) and centrifuged for 5 min (13,400×g). Again, the supernatant was removed and the pellet was incubated in a lysozyme-lysostaphyn (0.1 mg–0.01 mg) mixture for 15 min at 37°C. After this pre-treatment, DNA isolation was performed with the QIAamp DNA Mini Kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). Finally, DNA was eluted in 100 μl of nuclelease-free H2O. All samples were processed including isolation and amplification controls.

**Multiprobe Assay**

The primers and the universal bacterial TaqMan probe have been described previously [11]. The probes for *Pseudomonas* spp., *P. aeruginosa*, *E. coli*, *Staphylococcus* spp., *S. aureus*, *Enterococcus* spp., *Streptococcus* spp. were previously designed and described [10]. Each test contained 5 μl purified sample and 20 μl reaction mixture. The reaction mixture contained 12.5 μl of Taqman Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA, USA), 0.9 μM of forward primer, 0.6 μM of reverse primer, and 0.2 μM of each probe. There were four separate reactions: I) universal probe, *Pseudomonas* spp. probe and *E. coli* probe, II) *Pseudomonas aeruginosa* probe, III) *Staphylococcus* spp. probe, *S. aureus* probe and the *Enterococcus* spp. probe, IV) *Streptococcus* spp. probe. Reactions were performed on the ABI PRISM® 7900 real-time PCR System (Applied Biosystems, Foster City, CA, USA) and optimal thermal cycling conditions were as follows: 10 min at 50°C, initial denaturation at 95°C for 15 min, 42 cycles of denaturation for 15 s at 95°C and annealing at 60°C for 1 min. Cycle threshold (Ct), the cycle number at which amplicon fluorescence exceeded the preset detection threshold, was recorded for all samples. The threshold for the Ct analysis was manually adjusted to 0.1, together with the baseline start and end (cycle):6–15.

| Cut-off value | Sensitivity | Specificity | PPV | NPV |
|--------------|-------------|-------------|-----|-----|
| 24           | 92          | 83          | 77  | 95  |
| 25           | 97          | 80          | 75  | 98  |
| 26           | 97          | 73          | 69  | 98  |
| 27           | 98          | 65          | 63  | 98  |
| 28           | 98          | 59          | 59  | 98  |
| 29           | 98          | 54          | 57  | 98  |
| 30           | 100         | 48          | 54  | 100 |

Ct 25 was used as definitive cut-off value.

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**Figure 1. Receiver Operator Characteristic (ROC) decision plot.**

ROC curve analysis obtained by using the real-time PCR Ct values (universal probe) versus urine culture results (Cut-off value of ≥10^3 CFU/ml).

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Statistical Analysis
The positive predictive value (PPV), negative predictive value (NPV), sensitivity and specificity were calculated for the real-time PCR using the presence of ≥10^5 CFU/ml in culture as gold standard. For the statistical analysis, data were analyzed using SPSS software, version PASW Statistics 18 (SPSS Inc, Chicago IL, USA). Receiver Operator Characteristics (ROC) curve analysis was performed to determine a real-time PCR-based semi-quantitative breakpoint. The Mann-Whitney U Test and Kruskal-Wallis Test were used to enable correlation of the PCR Ct values with the bacterial load determined in culture. A p-value of <0.05 was considered significant.

Results
Population and Assay Characteristics
The collection of urine specimens consisted of 330 samples, of which 279 samples were analyzed retrospectively using the multiprobe assay. Six samples were excluded because of insufficient sample volume, 43 urine specimens were determined as skin/mixed flora and considered contaminants and 2 samples were excluded because of a fungal infection. In our test collection, the prevalence of a positive urine culture was 38%. The complete assay including DNA extraction and real-time PCR could be performed within 4 hours.

A Semi-quantitative Breakpoint for the Detection of Significant Bacteriuria
Within our University Medical Center, urine samples (in conjunction with the matching clinical symptoms) are categorized into two groups: UTI if bacteria are present at a concentration equal or higher than 10^5 CFU/ml and no UTI if the concentration of the bacteria is less than 10^5 CFU/ml. In this study, we also wanted to determine a semi-quantitative real-time PCR-based cut-off value to distinguish between UTI and no UTI. Therefore, the universal 16S rDNA probe was used as target, and Ct values were related to the cut-off value applied in culture. To evaluate the accuracy and the discriminating power of our diagnostic test, a ROC curve was made (Figure 1). The real-time PCR assay showed a good accuracy (AUC = 0.93). Following, the threshold used to discriminate between positive and negative urine samples was set to a Ct value of 25. At this threshold, 103 samples with UTI were found to be PCR positive and 3 samples negative, whereas 138 samples without UTI were PCR negative and 35 samples were found to be false PCR positive. This resulted in a significant difference in Ct value between the UTI and no UTI group (Figure 2). Using this cut-off value, a sensitivity and specificity of 97% and 80% respectively, could be reached. The positive and negative predictive values were 75% and 98%, respectively (Table 1).

As shown in Figure 2, two culture-positive samples were false-negative in our diagnostic PCR assay (Ct value of 29.56 and 26.62). An internal amplification control showed that the late
signals were not due to inhibition (data not shown). The first sample contained *Enterococcus faecalis* (10⁵ CFU/ml), and the second *Enterobacter cloacae* (≥10⁵ CFU/ml). Furthermore, we observed thirty-five false-positive results with PCR, of which in five cases the culture report mentioned the presence of a specific pathogen between 10⁴ and 10⁵ CFU/ml. In the remaining thirty cases no pathogens were found in culture. Adjusting the cut-off value to Ct 30 could resolve the two false-negative results. However, then the amount of false-positive isolates would increase to 90 cases. In Table 1, a summary is given of the performance characteristics of the PCR assay using alternative cut-off values.

In different guidelines as well as in specific population groups, cut-off values of 10³–10⁴ CFU/ml may be considered for the definition of UTI. In Figure 3, the Ct values of the universal probe were scattered against the bacterial load determined in culture. The results for low-level bacteriuria (10³–10⁴ CFU/ml) show all but one sample have Ct values of ≥30. If one would consider samples with ≥10³ CFU/ml as UTI and samples below 10³ CFU/ml as no UTI, and uses Ct 30 as a semi-quantitative breakpoint, the assay would show a sensitivity of 97%, specificity of 87%, positive predictive value of 85% and negative predictive value of 97%.

**Molecular Probes for the Identification of the Most Prevalent Uropathogens**

The real-time PCR assay was, in addition to the universal 16S rDNA probe, complemented with seven genus- or species-specific probes. In this way, parallel to the differentiation between UTI and no UTI with the eubacterial 16S probe, the multiprobe assay also offered a first identification of clinically relevant uropathogens. We analyzed 279 urine specimens, of which 92 samples were considered as significant bacteriuria because of the presence of 10⁵ CFU/ml of a single uropathogen (Table 2. I). The Ct value derived from the universal 16S rDNA probe was used as reference point. If no other probe generated a signal within 4 Ct proximity of the universal probe, the pathogen was identified as ‘other’, being a pathogen not included in the bacterial panel. The panel of probes could identify 78% of the pathogens present in the samples with monomicrobial infections. *E. coli* was the most frequently found uropathogen (62%), and was 100% correctly identified with the specific probe. The other probes targeting *Staphylococcus* spp., *Enterococcus* spp., *Pseudomonas* spp., *Pseudomonas aeruginosa* also showed a detection rate of 100%. One sample containing a *Streptococcus* spp. was missed, resulting in a detection rate of 80%. In fourteen isolates, a polymicrobial infection was shown (Table 2. II). The assay was able to identify more than one microorganism within the same sample. As shown in Table 2 (III), in sixteen cases pathogens were found both in culture and in PCR, but were, because of the presence of low counts, considered as no UTI. Finally, 43 samples were excluded from the study due to containing contaminating skin/mixed flora as determined by conventional diagnostics. PCR analysis was performed on a subset of 11 of these samples. Results showed that in agreement with the presented data, bacterial counts could be semi-quantified and the assay used for identification. In 1 of 11 cases, the assay showed a signal for *E. coli* within 4 Ct of the universal probe, in contrast to

![Figure 3. Boxplot showing the correlation of PCR Ct values with bacterial load determined in culture. Culture results were grouped into three categories: <10³ CFU/ml, 10³–10⁴ CFU/ml and ≥10⁵ CFU/ml. Statistical significance was determined performing Kruskal-Wallis Test. doi:10.1371/journal.pone.0061439.g003](image-url)
culture data. In all remaining samples, different combinations of probes generated signals but all at high Ct values, more than 4 Ct later than the universal probe, in agreement with the culture findings of a mixed flora (data not shown).

### Discussion

In this study, we present a novel molecular approach which utilised all capabilities of broad-spectrum real-time PCR to aid in the diagnosis of UTIs. The assay detecting the majority of uropathogens as well as a universal probe detecting eubacteria was used to distinguish urinary samples with significant, or low or no bacteriuria. Secondly, this screening assay was reinforced with...
mended colony counts of the American Society for Microbiology (ASM) and European urinalysis guidelines have recommended that any colony count above 10^5 CFU/ml be considered indicative of a UTI. The results showed that lowering the culture cut-off to 10^3 CFU/ml in order to establish a discriminatory set point between bacteriuria and no bacteriuria has been the Sysmex UF-1000i criterion for detecting bacteriuria. In order to prevent positive samples to be falsely classified as bacteriuria, we performed amplification experiments: WH CvdD PW. Analyzed the data: WH PW. Wrote the paper: WH CvdD PW. Revised the paper: WH PW.

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**Author Contributions**

Informed consent: WH CvdD PW. Preparation of the manuscript: WH CvdD PW. Data collection: WH CvdD PW. Statistical analysis: WH CvdD PW. Preparation of the manuscript: WH CvdD PW.
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