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Applied Microbiology: Open Access

Research Article

Comparison of Multiplex Real-Time PCR with Standard Urine Culture for Identification of Pathogens Associated with Canine Urinary Tract Infections

Samantha J Guida[1*], Joseph Bartges[1], Rebekah Jones[2], Caleb Young[3], Maria Cekanova[3]

[1]Department of Small Animal Medicine and Surgery, The University of Georgia, Georgia, USA; [2]Department of Biomedical and Diagnostic Services, University of Tennessee, Tennessee, USA; [3]Integrity Laboratories, Knoxville, Tennessee, USA

ABSTRACT

Conventional aerobic microbial urine culture with antimicrobial susceptibility testing (UCS) is reference standard for diagnosis of canine Bacterial Urinary Tract Infections (BUTIs). Emerging multiplex real-time PCR (qPCR) may be a useful diagnostic test when performed in conjunction with UCS. The aim of this study was to compare qPCR with UCS for identification of uropathogens in canine urine. Twenty-three frozen isolates of canine uropathogens were selected from an archive and grown on blood agar plates. Following growth on blood agar, colonies from each isolate were inoculated into sterile canine urine, creating 23 positive contrived urine specimens. Urine specimens were incubated for 40 hours at 38°C. Samples were split into two sets of 23 specimens; the first set was analyzed by UCS, and the second set was analyzed by qPCR. Two specimens of sterile urine were used as negative controls. Blind urine sample testing was performed at both analytical laboratories.

UCS correctly identified uropathogens in 22 of 23 positive isolates. qPCR correctly identified uropathogens in 20 of 23 isolates. Controls did not yield bacterial growth. One sample containing *Staphylococcus schleiferi* was identified by UCS, but not qPCR; however, qPCR detected unspecified uropathogen by presence of 16S RNA. In 3 samples, qPCR identified an additional organism that was not detected by UCS. qPCR had comparable results to UCS for identification of canine uropathogens from frozen isolates. If similar results are seen in urine from dogs with naturally occurring urinary tract infections, qPCR may serve as a useful adjunctive diagnostic tool.

Keywords: Antimicrobial resistance; Urine culture; Real time polymerase chain reaction; Uropathogens

INTRODUCTION

Bacterial Urinary Tract Infections (BUTIs) are a common cause of lower urinary tract signs in dogs and are among the leading reasons for antimicrobial therapy [1]. Prevalence rates of BUTI in dogs have been reported as 14% to 26.6% in females and 6.2% in males [2]. The reference standard for diagnosing a BUTI is conventional aerobic microbiological urine culture with antimicrobial susceptibility testing (UCS) [1,3]. While UCS is reliable, results may take several days to isolate the aerobic microbe and determine antimicrobial susceptibility [3]. Therefore, it is common practice in both human and veterinary medicine to begin empiric antimicrobial therapy while UCS results are pending [1,3].

In human medicine, evidence is accumulating supporting use of molecular techniques, such as real-time Polymerase Chain Reaction (qPCR), as an adjunct tool for diagnosing BUTIs. Multiplex qPCR testing utilizes multiple primers to detect several targets at the same time [3]. Advantages of PCR-based testing for BUTIs include its high sensitivity and specificity, as well as the rapidity with which results can be obtained [3-6]. While UCS is limited to detection of secondary pathogens, PCR is able to detect an unlimited number of pathogens based on present genetic material [3]. In addition, qPCR determines not only qualitative analysis of pathogens, but also provides quantitative information on the number of detected pathogens and their ratio [7]. This is an important consideration, as polymicrobial BUTIs may occur more frequently than believed, and display enhanced virulence and poly-antimicrobial resistance [3]. Appropriate treatment depends on accurate identification of the causative organisms.

While qPCR appears to have a role for diagnosis of BUTIs in humans, this technique has yet to be evaluated for diagnosis of canine or feline BUTIs. However, the use of multiplex qPCR has been described in veterinary medicine for other purposes,

Correspondence to: Samantha J Guida, Department of Small Animal Medicine and Surgery, The University of Georgia, Georgia, USA, Tel: +14015788588; Email: sguida@uga.edu

Received: 03-Apr-2023, Manuscript No. AMOA-23-22807; **Editor assigned:** 05-Apr-2023, Pre QC No. AMOA-23-22807(PQ); **Reviewed:** 21-Apr-2023, QC No. AMOA-23-22807; **Revised:** 01-May-2023, Manuscript No. AMOA-23-22807(R); **Published:** 09-May-2023, DOI: 10.35284/2471-9315.23.9.253

Citation: Guida SJ, Bartges Y, Jones R, Young C, Cekanova M (2023) Comparison of Multiplex Real-Time PCR with Standard Urine Culture for Identification of Pathogens Associated with Canine Urinary Tract Infections. Appli Microbiol Open Access. 9:253.

such as for diagnosis of *Leptospira spp*., *Brucella canis*, and canine distemper virus [8-10]. The present study was conducted to evaluate the potential utility of multiplex qPCR for diagnosis of canine BUTIs. The objective was to compare qPCR with UCS for detection of 23 canine uropathogens, using frozen isolates selected from an archive. We hypothesized that qPCR-based testing would match results from UCS testing of stored canine uropathogens and negative controls.

MATERIALS AND METHODS

Selection of uropathogen isolates

Twenty-three positive culture isolates of canine uropathogens were selected from an archive containing a total of 751 isolates of canine and feline uropathogens stored at -80°C. These isolates were collected from clinical urine specimens from dogs and cats diagnosed with BUTI at University of Tennessee, Knoxville (UTK) College of Veterinary Medicine from January 1st 2008 to December 31st 2008.

Inoculation of uropathogens on blood agar plates

The 23 selected uropathogen isolates were removed from -80°C storage and transitioned to a freezer block. Each of the 23 isolates was inoculated onto a blood agar plate (5% Sheep Blood in Tryptic Soy Agar Base, Hardy Diagnostics) with their respective number. For inoculation, ice crystals were scraped using an 18-gauge 1.5-inch needle, and the end of the needle was then lightly touched to an agar plate. The plate was then streaked with a sterile inoculating loop (Fisherbrand Disposable Inoculating Loops, Fisher Scientific), using the 4-quadrant streak plate technique [11]. The 23 plates were incubated (MyTemp Mini Digital Incubator 2013, Benchmark Scientific) at 38°C for 24 hours.

Urine sample collection

Urine was collected from a healthy 6-year-old male neutered Great Dane using clean technique. An 8-Fr red rubber catheter was inserted transurethrally after cleaning the extruded penis with surgical scrub and sterile saline, and 60 mL of urine was obtained. The urine was stored in sterile culture tubes, and a sample was submitted to the University of Georgia (UGA) Diagnostic Laboratory to prove sterility (absence of bacterial organisms). The urine was then aliquoted into 25 cryotubes (Cryogenic vials, Corning), containing 1.5 mL of urine each.

Inoculation of urine samples

The 23 blood agar plates were evaluated for bacterial growth after 24 hours of incubation at 38°C. Plates that showed bacterial growth were used for inoculation of sterile canine urine. For inoculation of sterile urine, each plate was swabbed with a sterile inoculating loop, which was then dipped in their respective urine aliquot. The urine specimens were incubated at 38°C for 40 hours. The blood agar plates were stored and refrigerated.

Standard Urine Culture (UCS) and Real-time Polymerase Chain Reaction (qPCR)

Tubes with pathogen-inoculated urine were removed from the incubator after 40 hours. Each of the 23 urine tubes, containing 1.5 mL each, was divided into 2 sterile tubes: one tube containing 0.5 mL sample and one tube containing 1 mL sample. This process was repeated for two cryotubes containing only sterile urine (negative control samples without inoculated uropathogens). The 0.5 mL samples underwent standard UCS at UGA Diagnostic Laboratory. The 1 mL samples were shipped overnight to Integrity Laboratories and analyzed using multiplex qPCR. Both laboratories were blinded during testing of the 25 samples.

Mulitplex qPCR

The urine samples were tested using K9UTIID test (Integrity Laboratories, LLC, Knoxville, Tennessee), a laboratory-developed multiplex qPCR designed to detect deoxyribonucleic acid (DNA) of pathogens present in urine specimens. This includes detection of the following: *Candida albicans*; *Citrobacter freundii*; *Clostridium perfringens*; *Corynebacterium urealyticum*; *Enterobacter cloacae*; *Enterobacter aerogenes*; *Enterococcus faecium*; *Enterobacter faecalis*; *Escherichia coli*; *Klebsiella oxytoca*; *Klebsiella pneumoniae*; *Mycoplasma hominis*; *Proteus mirabilis*; *Proteus vulgaris*; Pseudomonas aeruginosa; *Serratia marcescens*; *Staphylococcus aureus*; *Staphylococcus pseudintermedius*; *Streptococcus agalactiae*. In addition, the K9UTIID test detects specific genes in bacteria causing the most common antibiotic-resistance: *CTX-M*, *KPC*, *NDM1*, *mecA*, *dfrA*, *sul1*, and *qnrS* (not included in data analysis).

Once received, the urine specimens were stored in a refrigerator until qPCR was performed. A small aliquot of each sample was pipetted into a 96-well microplate to extract DNA, and 2 µL of extracted DNA specimen was mixed with qPCR master mix containing primers and probes in a 384-well microplate. qPCR reaction was performed using QuantStudio™12K Flex Real Time PCR System (ThermoFisher Scientific Inc.). The housekeeping gene, canine glyceraldehyde-3-phosphate dehydrogenase (K9GAPDH), was used as an internal positive control to confirm presence of canine DNA from uroepithelial cells. The housekeeping gene for detection of bacterial 16S ribosomal RNA (16S rRNA) was used as a positive internal control to confirm the presence of bacteria in urine. 16S rRNA was used to avoid false negative results for uropathogens that were not specifically identified by the K9UTIID test. Canine sterile urine negative matrix specimens were used as internal negative matrix controls for qPCR. All positive and negative controls were used to confirm conditions for each step of qPCR and verify the results of tested specimens by qPCR. The results with identified uropathogens (primary and secondary) in tested urine specimens were sent to UGA for data analysis.

Standard USC at UGA

The urine samples were analyzed using aerobic microbial culture technique at UGA. Refrigerated urine samples were plated on Tryptic Soy Agar with 5% sheep blood (BD Diagnostics), MacConkey Agar (Remel), and Columbia CNA Agar (Remel). All plates were incubated at 35°C with 5% CO2 for 48 hours. After a cytospin preparation using 100 microliters of urine, the slides were heat fixed and a Gram stain was performed. Plates were observed at 24 and 48 hours. Cultures with no growth after 48-hour incubation were concluded as specimens with no bacterial growth. If bacterial growth was present, the plate underwent colony count, identification, and susceptibility testing.

Statistical methods

Positive and Negative Percentage Agreement (PPA and NPA) were determined for UCS and qPCR test results, as compared to original results of archived uropathogens at UTK. Data were analyzed using descriptive statistics.

RESULTS AND DISCUSSION

Evaluation for bacterial growth on blood agar plates

All 23 plates inoculated with archived bacterial isolates showed positive bacterial growth after 24 hours (Table 1).

Table 1: Identification of primary and secondary uropathogens by standard aerobic microbial urine culture with antimicrobial susceptibility testing (UCS) and multiplex real-time Polymerase Chain Reaction (qPCR) compared to original urine culture results.

Canine uropathogen	Number of identified primary uropathogens			Urine culture: Colony count & gram stain findings	Number of identified secondary uropathogens	
	Original urine culture	UCS	qPCR		UCS	qPCR
E. coli	10	10	10	>100,000 cfu/µL	1 *S. canis* 20,000 cfu/uL	1 *C. perfringens*
Staphylococcus pseudintermedius	3	3	3	>100,000 cfu/µL	0	1 *E. coli*
Enterococcus	2	2	2	>100,000 cfu/µL	0	1 *E. coli*
Pseudomonas aeruginosa	3	3	3	>100,000 cfu/µL	0	0
Serratia marcescens	1	1	1	>100,000 cfu/µL	0	0
Staphylococcus. schleiferi	1	1	0	>100,000 cfu/µL	0	0
Klebsiella pneumoniae	1	1	1	>100,000 cfu/µL	0	0
Proteus mirabilis	2	1	0	1,000 cfu/µL Gram negative *bacilli* seen	0	0

Note: cfu: Colony forming units; UCS: Aerobic microbial urine culture with antimicrobial susceptibility testing; qPCR: Multiplex real-time polymerase chain reaction.

Standard Urine Culture (UCS)

UCS correctly identified 22 of the 23 positive uropathogens and correctly did not grow bacteria from the 2 controls (Table 1). UCS yielded >100,000 colony forming units (cfu)/µL bacterial growth in 21 of 23 samples. *Proteus mirabilis* was correctly identified in one of two samples at 1,000 cfu/µL. The second sample did not grow *Proteus mirabilis* but did identify gram negative *bacilli* on gram stain. One *E. coli* sample was correctly identified by UCS; however, a secondary pathogen, *Streptococcus canis*, was also detected at 20,000 cfu/µL.

Multiplex qPCR

Multiplex qPCR correctly detected and identified the presence of uropathogens in 20 of 23 of positive specimens, and correctly identified the two negative controls (Table 1). qPCR failed to identify *Proteus* sp. in two urine specimens. Bacterial 16S rRNA and K9GAPD also failed to be detected in the two *Proteus* specimens. The sample inoculated with *Staphylococcus schleiferi* was only identified by the presence of 16S rRNA in urine (true positive results) due to absence of specific primers and probes in the qPCR test. Internal control for K9GAPDH gene was not detected in the urine specimen inoculated with *Pseudomonas aeruginosa* (n=1) and *Proteus mirabilis* (n=2) due to low amount of tested canine gene in sample.

Comparison of UCS (UGA) and qPCR (Integrity Laboratories) to original results from archived specimens obtained from UTK

Twenty out of 23 positive urine specimens were correctly identified by both UCS and qPCR. The urine sample inoculated with *Staphylococcus schleiferi* was only identified by UCS, but not by multiplex qPCR due to absence of specific primers and probes. However, this specimen was tested positive for the presence of uropathogen by 16S rRNA in urine (true positive results) by qPCR. Of the two samples containing *Proteus mirabilis*, only one specimen was correctly identified by UCS, and neither was identified by qPCR. 16S rRNA was not identified in urine specimens with *Proteus mirabilis*.

In three samples, multiplex qPCR identified secondary uropathogens that were not detected by UCS test results at UGA or UTK: *E. coli* (secondary) in sample with *Enterococcus faecium* (primary), *Clostridium perfringens* (secondary) in sample with *E. coli* (primary), and *E. coli* (secondary) in sample with *Staphylococcus pseudintermedius* (primary). UCS identified *S. canis* as a secondary uropathogen in the specimen inoculated with *E. coli*.

PPA and NPA

Compared to original culture results of archived uropathogens, the PPA and NPA for UCS were 100% and 66.67%, respectively. PPA and NPA for multiplex qPCR were 100% and 40%, respectively. Overall PPA and NPA agreements between UCS and multiplex qPCR on identification of primary uropathogens were 100% and 50%, respectively. UCS correctly identified 22 of 23 samples of primary uropathogens, whereas qPCR correctly identified primary uropathogens in 20 of 23 samples. UCS and qPCR tests detected all samples with *E. coli* (n=10) with 100% accuracy, and this organism represents the most common isolate in canine BUTIs [2,12]. *Staphylococcus schleiferi* (primary uropathogen in one sample) was not included in the menu of tested uropathogens by qPCR, which explains why it was detected by UCS and not qPCR. However, this specimen tested positive for internal positive control, bacterial 16S rRNA, by qPCR.

In four of the 23 specimens, a secondary bacterium was identified in addition to the primary uropathogens. qPCR but not UCS identified *E. coli* in two specimens and *Clostridium perfringens* in one specimen. UCS but not qPCR identified *Streptococcus canis* in one specimen, but this organism was not included in the menu of tested uropathogens by qPCR. The significance of the secondary pathogens detected by qPCR (and not UCS) is unknown. One consideration is contamination of the urine samples during transit of urine samples to the laboratory performing qPCR. Contamination is unlikely to have occurred prior, as it would be expected that UCS would have also detected the pathogens in that case. Similarly, it is unlikely that the secondary pathogens were present in the urine collected from the dog in this study, as UCS failed to identify them, and the controls were negative as well.

In this study, UCS and qPCR failed to detect *Proteus mirabilis* adequately, which was the primary uropathogen inoculated in

two samples. UCS correctly identified *Proteus* in one sample, but in low numbers (1,000 cfu/μL). The second sample containing *Proteus* had no growth on UCS; however, it did identify gram negative *bacilli* on gram stain, which is consistent with the classification of this bacterium [13]. qPCR did not identify *Proteus mirabilis* in either of the two samples. For this reason, a follow-up experiment was performed, with six additional samples tested by UCS and qPCR. All six samples grew high numbers of *Proteus mirabilis* colonies on UCS, concluding that the low growth noted in the initial experiment may be attributed to faulty technique. However, all six samples tested negative for *Proteus mirabilis*, as well for 16S RNA by qPCR. This suggests that there may be something in the original specimens that is inhibiting the qPCR reaction.

The *K9GAPDH* gene was not detected in three urine samples. *K9GAPDH* is a housekeeping gene to confirm presence of canine DNA of tested specimens and served as internal control for qPCR test. The absence of *K9GAPDH* does not affect the diagnosis of BUTI in tested specimens, as qPCR tests for specific pathogen's DNA/RNA. In addition, the absence of the housekeeping genes is a common finding in urine, particularly in male dogs due to low number of released urothelial cells in urine [14].

In human medicine, qPCR is considered a rapid, accurate, and available tool for diagnosis of BUTIs, helping guide early treatment decisions by including genes commonly associated with drug-resistance in urinary pathogens [3,15]. Nevertheless, the qPCR method is a tool to be used in conjunction with, and not as a replacement for UCS, as several disadvantages exist [3]. One downside of qPCR is its ability to identify clinically insignificant pathogens, including dead organisms, pathogens that are present in urine, or pathogens that are a part of the urinary microbiome [16]. In addition, unlike UCS, qPCR results do not provide information of antimicrobial sensitivity, which is essential for antibiotic stewardship [3,17]. Disadvantages that may particularly apply to veterinary medicine include the increased cost associated with qPCR and limited availability of laboratories to perform qPCR, both of which may affect its utility in a clinical setting. Another limitation of qPCR assay is that some pathogens may not be part of the detection panel, as seen with *Streptococcus canis* and *Staphylococcus schleiferi* in this study.

While susceptibility results were performed in this study, results were not provided. The comparison of antimicrobial resistance genes with resistance patterns on UCS was not possible due to the paucity of primers for qPCR. Additionally, susceptibility patterns differed between original UCS and those performed as part of the study due to use of different antimicrobial tested. This is another limitation of the study, as it would have been useful to evaluate antimicrobial data and compare susceptibility patterns between diagnostic laboratories. Further development and validation of antimicrobial resistance genes compared with UCS results is warranted.

CONCLUSION

The results of this study support that qPCR had comparable results to UCS in detection of canine uropathogens. Because multiplex qPCR has yet to be validated for diagnosis of canine BUTIs, frozen isolates were utilized for this research so identification of each uropathogen was already predetermined. The 23 uropathogens were also recultured to confirm viability prior to study testing by UCS and qPCR. Based on the results and study design, we conclude that qPCR was able to identify canine uropathogens from frozen isolates. Additional research is needed to determine whether this method is useful in a clinical setting. Future prospective studies may include testing with both UCS and qPCR on urine from dogs with naturally occurring BUTIs, with and without clinical signs.

DECLARATION OF COMPETING INTEREST

Cekanova and Young are employees of Integrity Laboratories, LLC in Knoxville, TN but were not involved in study design or data analysis. Tested specimens for qPCR test were blinded. No other authors have a conflict of interest.

ACKNOWLEDGEMENTS

This work was supported in part by Integrity Laboratories (performed quantitative PCR at no charge) and the Department of Small Animal Medicine and Surgery at The University of Georgia College of Veterinary Medicine. The funders had no role in study design or data analysis. Preliminary results were presented as a poster at the 4th Clinical and Scientific Advances in Urinary Tract Infection Conference, virtual, 19 July 2022. We wish to thank Kathleen Hoover (University of Georgia), as well as Amy McKinney and Ashley Phillips (Athens Diagnostic Laboratory) for their technical support.

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