Quantification of Bacteriuria Caused by Hemolysin-Positive *Escherichia coli* in Human and Mouse Urine Using Quantitative Polymerase Chain Reaction (qPCR) Targeting *hlyD*

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Abstract

Uropathogenic *Escherichia coli* (UPEC) cause the majority of community-acquired urinary tract infections (UTIs). Quantitation of bacteriuria (the number of bacteria in urine) is important for diagnostic approaches and in diverse research applications. Most UPEC strains express hemolysin, the expression of which has been correlated with the severity of UTI in murine models of infection. In this study, we sought to develop and optimise a quantitative Polymerase Chain Reaction (qPCR) assay for enumeration of hemolysin-positive UPEC in urine. Using recombinant plasmid pJET1.2::*hlyD*, which we termed pGU2470, the sensitivity range and linearity of amplification of qPCR was determined using primers and a probe targeting *hlyD*. Whole-cell preparations containing UPEC were quantified for *hlyD* copy number using C_T values and compared to standards prepared with a known amount of pGU2470. We compared the efficiency of the assay for analysis of human and mouse urine because mouse models of human UTI are frequently used for investigating UPEC UTI. Urine samples were collected from healthy adults and mouse infection assays and used to assess any potential inhibitory effects of urine on the qPCR. The linear quantitative range of the qPCR (i.e. sensitivity) in detecting UPEC genomes was 10^3 copies/mL; qPCR-derived estimates of UPEC bacteriuria (based on number of genomes detected) were, on average, ten-fold higher than culture-based estimates. Finally, the frequencies of positive and negative predictions of UPEC in urine using the qPCR were equivalent to colony count methods. This assay provides an alternative to culture-based approaches for quantitation of UPEC bacteriuria in research studies.

Keywords: Urinary tract infection; *Escherichia coli*; bacteriuria; quantitative polymerase chain reaction; urine

Introduction

Urinary tract infections (UTI) are one of the most common human bacterial infections, costing \$1.6 billion per year (Chu and Lowder, 2018, Flores-Mireles et al., 2015). These infections are particularly prevalent in women, with over 1 in 10 women presenting to a doctor with a UTI per year; 20-30% of these women will experience recurrent UTI (Chu and Lowder, 2018). Uropathogenic *Escherichia coli* (UPEC) cause the majority (up to 75%) of community-acquired UTI (Terlizzi et al., 2017). Quantitation of UPEC in urine provides insight into the severity of UTI and is routinely performed using bacteriological culture. A few studies have applied molecular approaches to detect bacteria in urine (Ipe et al., 2013) but none are routinely used in diagnostics, and few have appeared in research studies. Bacteriological culture approaches are limited to the quantitation of live, cultivable bacterial cells, and several species that are associated with UTI can grow in urine (Ipe et al., 2016).

UPEC strains are classified into four main phylogenetic groups (A, B1, B2, D), and can contain a variety of pathogenicity islands and virulence factors, such as capsule, pillus and non-pilus adhesins, flagella, toxins, and nutrient acquisition systems (Terlizzi, Gribaudo and Maffei, 2017). UPEC virulence factors enable evasion of innate immune mechanisms and contribute to UTI pathogenesis (Ulett et al., 2013). The pore-forming toxin hemolysin (HlyA), that lyses red blood cells, is produced by many UPEC strains; its expression correlates with the severity of UTI, as reviewed elsewhere (Ristow and Welch, 2016). UTI severity is frequently studied using mouse models of human infection, which have been used extensively to help to elucidate

the mechanisms of UPEC virulence and host-pathogen interactions (Carey et al., 2016). A molecular method to quantify UPEC bacteriuria in mice would be valuable for a variety of research studies to circumvent the limits of bacteriological culture, such as the inability to detect non-cultivable bacteria, and to allow relatively quicker processing times compared to culture.

Hemolysin is encoded in the genomes of less than 15% of commensal *E. coli*; in contrast, half of cystitis-causing UPEC strains, and three-quarters of UPEC strains that cause pyelonephritis, carry the gene encoding hemolysin, *hlyA* (Ristow and Welch, 2016). The cytotoxic effects of HlyA are dose-dependent and, in macrophages, include triggering of inflammasome responses (Murthy et al., 2018, Schaale et al., 2016). HlyA causes exfoliation of bladder epithelial cells (Ristow and Welch, 2016) and disrupts kidney function, which may impede urine flow and allow bacterial ascension to cause pyelonephritis (Terlizzi, Gribaudo and Maffei, 2017).

The *hlyA* gene is contained within the *hlyCABD* operon; *hlyA* encodes the effector protein; *hlyC* is required for post-translational activation of HlyA; *hlyB* encodes an ABC transporter, and *hlyD* encodes an outer membrane-forming channel protein (Ristow and Welch, 2016). The *hlyCABD* operon is present in most UPEC isolates positive for pap adhesion, which includes the frequently studied CFT073 and UTI89, both with one copy of the hemolysin operon. Reference strains J96 and UPEC 536 also express hemolysin, and contain two *hly* operons (Maslow et al., 1995, Swenson et al., 1996, Wiles et al., 2008). Recently, Velasco et al, 2018 reported differential expression of the *hly* operons of J96 under certain conditions (Velasco et al., 2018). In addition, the *hlyCABD* operon was identified in pathogenic *E. coli* O83:K24:H31 (Sheshko et al., 2006). In this study, we sought to develop and optimise a qPCR

assay for the quantification of UPEC in urine for research applications focused on bacteriuria, particularly in mice.

Materials and Methods

Ethics

Approval for animal experimentation was granted by the Griffith University Animal Ethics Committee (MSC/14/08AEC). Approval for use of urine samples from adult human volunteers was granted by the Griffith University Human Ethics Committee (MSC/11/10/HREC).

Bioinformatics and oligonucleotide design

NCBI BLAST was used to search reference bacterial genomes for homologs of *hlyD* (locus tag: c3574) from hemolysin-positive UPEC CFT073 (Mobley et al., 1990).

Identical nucleotides in UPEC *hlyD* were compared to homologous sequences from seven other UPEC genomes, and fourteen other bacteria (n=21) using ClustalW alignment in Molecular Evolutionary Genetics Analysis (MEGA6) software. The choice of targets for primer design was based on identification of highly conserved regions amongst UPEC strains, which were sufficiently divergent from other bacteria that contained homologs of *hlyD*. The primers designed for UPEC-specific regions were: *hlyD*_F 5-CCTGAACTGAAGCTTCCTGA-3; *hlyD*_R 5-CGGGCAAGTATTGTTAATCG-3. Primer 3 Plus (Untergasser et al., 2012) was used to design the following probe targeting *hlyD* for quantitation: *hlyD*_P 5-TCCACATGGCAAATCAGAA-3.

Oligonucleotide primers and the hydrolysis probe (labelled with 6-FAM at the 5' end, and BHQ1 at the 3' end) were purchased from Sigma Aldrich.

Bacteria

UPEC strain, CFT073 (Mobley, Green, Trifillis, Johnson, Chippendale, Lockatell, Jones and Warren, 1990) and *E. coli* DH5 α were routinely grown in lysogeny broth (LB) with agitation at 200 rpm, or on LB agar overnight at 37°C. For some experiments, CFT073 was grown in synthetic human urine (SHU) that was prepared as previously described (Ipe and Ulett, 2016).

Genomic DNA isolation, PCR and *hlyD* purification

Genomic DNA (gDNA) was isolated from UPEC CFT073 using methods as described previously (Sambrook and Russell, 2001). Plasmid DNA was isolated using QIAprep spin miniprep kits (QIAGEN). All DNA was quantified using a NanoDrop-1000 spectrophotometer and stored at -20°C. Polymerase Chain Reaction was used to amplify the *hlyD* region using primers *hlyD__F* and *hlyD_R* and Phusion high fidelity polymerase according to the manufacturer's specifications (New England Biosciences), with the following cycle conditions: Initial denaturation 98°C 30 s, 35 cycles (98°C 10 s, 60°C 30 s, 72°C 15 s), final extension 72°C 5 minutes. PCR products were separated using an 0.8% agarose gel and the 170bp *hlyD* amplicon was purified using a QIAquick Gel extraction kit (QIAGEN).

Cloning, transformation and plasmid copy number

To construct a plasmid containing *hlyD* sequence for use as a positive control and DNA standard, a 170 bp *hlyD* amplicon (amplified using *hlyD_F* and *hlyD_R*) was cloned into pJET1.2 using the CloneJET PCR cloning kit (Thermo Fisher Scientific) according to manufacturer's instructions. DNA of recombinant pJET1.2::*hlyD* was sequenced using primers from the CloneJET kit, and termed pGU2470 (3,144 bp). OligoCalc software (Kibbe, 2007) was used to define the pGU2470 dsDNA molecular weight (MW) as 1942403.8g/mol; this MW, along with Avogadro's number and the

mass of the DNA in solution, was used in an equation derived from the one described in a prior study (Lee et al., 2006, Whelan et al., 2003) to calculate concentration (in ng. μl^{-1}) and prepare a solution of pGU2470 containing an estimated 10^9 copies. μl^{-1} .

Optimisation and sensitivity range of quantitative PCR (qPCR) for *hlyD*

A solution of 10^9 copies. μl^{-1} pGU2470 was used to prepare 10-fold serial dilutions (10^9 - 10^0 copies. μl^{-1}). LightCycler® 480 Probes Master mixes (Roche; catalogue 04707494001) were used in qPCR reactions on a Roche LightCycler® LC-480 II, according to manufacturers' instructions with a minor modification: 1 μl of template was used instead of 5 μl to minimize inhibitor interference. Primers and hydrolysis probes were optimized at 1 μM and 0.1 μM per reaction, respectively. The method for qPCR detection was: Mono-colour hydrolysis probe (6-FAM and BHQ1); Excitation 496nm, Emission 520nm; 96-well block and multiwell plate (catalogue 04729692001); reaction volume 20 μl . Cycling conditions were: Pre-incubation at 95°C for 5 min; 45 cycles of Amplification [95°C 10 s, 60°C 30 s, 72°C, 1 s], and 1 cycle of Cooling 40°C, 10 s. Fluorescence acquisition was post-72°C steps. Standard curves were generated using LightCycler® software and considered optimal when the amplification efficiency was in the range of 90-110% (Raymaekers et al., 2009).

Quantification of whole bacteria using qPCR

UPEC CFT073 was grown in SHU as described previously (Ipe and Ulett, 2016), and the number of colony forming units (CFU) was determined by plating on agar. Ten-fold serial dilutions were boiled for 10 minutes prior to using 1 μl as a template in the

qPCR reactions. Whole-cell preparations were quantified for copies of *hlyD* using C_T values and compared to standards that contained a known amount of pGU2470.

Mouse infection and urine sampling

10-12 week old female BALB/c mice (Animal Resources Centre, WA, Australia) were infected transurethrally with 50 μ l of PBS containing 5×10^7 CFU of CFT073, as previously described (Tan et al., 2012). At 2 h and 24 h, urine was collected. Low-volume samples (that were due to the main void being missed for some samples) were diluted with water up to 10 μ l. Small volumes of the samples were used for standard plate counts on LB agar, and remainders used for qPCR.

Inhibitory effect of urine on qPCR

In the absence of any qPCR inhibitory effects, reactions should exhibit a C_T difference of 2.32 cycles and 3.3 cycles between 5-fold and 10-fold serial dilutions, respectively (Cao et al., 2012). We used two approaches to assess the potential inhibitory effects of urine towards the qPCR. Firstly, 5-fold and 10-fold serial dilutions of urine sample collected from UPEC-infected mice were used as template following boiling lysis. Inhibition was assessed by the C_T difference. Secondly, we used 10^7 copies of pGU2470 spiked into serial dilutions of pooled mock-infected urine, to examine the effect of increasing urine concentrations on the C_T . The water component of qPCR reactions was replaced with the dilutions, such that qPCR reactions contained 6.8 μ l of a urine/water mix and a urine concentration ranging from 34% to 0.002% of the qPCR reaction. This was repeated using filter-sterilised human urine to compare human with mouse urine. The lowest dilution with the closest C_T to the positive control was accepted as the optimal dilution to avoid inhibition.

Comparing the quantification differences between qPCR and urine culture

Based on the results from the qPCR urine inhibition assays, mouse urine was serially diluted 1:10 and 1:100. Remaining samples were serially diluted for CFU determination on agar. Samples were prepared for qPCR preparation as outlined previously and run in triplicate. All diluted qPCR samples were back-calculated to the theoretical original value to compare to the respective CFU count on agar.

Statistical analysis

All statistics was performed using GraphPad Prism® version 5.

Results

In silico analysis of *hlyD* gene sequence for primer and probe design

To design primers and a hydrolysis probe that would be specific to *hlyD* of UPEC, using a bioinformatics approach we compared regions across the *hlyD* gene that are conserved amongst UPEC but absent from other bacteria that contain close (>50% identical) homologs of *hlyD*, as described in the methods section. BLAST analysis showed the sequences with most homology to UPEC-*hlyD* are present in other γ -proteobacteria in the order Pasteurellaceae, including *Aggregatibacter*, *Actinobacillus* and *Mannheimia*. We identified regions specific to UPEC strains that were absent from these other bacteria, and targeted these for use as oligonucleotide primers to generate a 170 bp *hlyD* amplicon (red lines, Figure 1), and a specific probe (blue line, Figure 1). BLAST analysis of several commensal *E. coli* strains (K-12 mg1655, SE11, SE15, HS, and Nissle 1917) returned no homologous sequences, and were not included in the alignment.

Generation of standard curve for *hlyD* using hydrolysis probe qPCR

Primers *hlyD_F* and *hlyD_R* were used to amplify the 170 bp *hlyD* amplicon and this was cloned into pJET1.2 to produce a plasmid containing *hlyD*, which we termed pGU2470. Using the same primers and hydrolysis probe *hlyD_P*, a 10-fold dilution series of pGU2470 starting at 10^9 copies. μL^{-1} was subjected to qPCR to generate a standard curve with known copy number. Optimal primer and probe concentrations were determined to be $1\mu\text{M}$, and $0.1\mu\text{M}$, respectively (data not shown). After several independent runs to verify the standard curve, the resulting primer efficiency was calculated at 101.9%, R^2 was 0.99, and the error was below 0.2 (Figure 2). This sensitive method was successful in detecting as few as 10 *hlyD* copies. The standard curve was utilised as an in-run calibrator to determine *hlyD* copy number in unknown samples.

Quantification of UPEC in prepared bacterial suspensions in SHU using qPCR

The *hlyD* probe-primer combination was applied to quantify UPEC in bacterial suspensions prepared in SHU, and the sensitivity of the qPCR assay was compared to the traditional quantification method of colony counts on agar to determine CFU. mL^{-1} . The results of plate count assays of UPEC cells that were grown in SHU to a density of $3\text{--}6 \times 10^6$ CFU. mL^{-1} and then serially diluted and plated onto LB agar are summarized in Table 1. qPCR results used to assess linear quantitative range, which were derived from samples of the same dilutions used for plate counts (and subjected to boiling lysis before $1\mu\text{L}$ being used as template in qPCR) are shown in Table 1. The data show that qPCR quantification based on whole-cell preparations was, on average, 10-fold higher than the CFU. mL^{-1} values obtained using the traditional plate count method. The average concentrations in copies/ml and CFU/ml (Table 1) represent two of five independent experiments, and define the linear quantitative range of the qPCR assay for UPEC cells as between $10^3\text{--}10^7$ copies/mL.

Specificity of *hlyD* primers in distinguishing UPEC from other common uropathogens

Using similar approaches to those above for whole-cells of UPEC, we tested the specificity of the *hlyD* primers in amplifying DNA from other common uropathogens. We cultured *Streptococcus agalactiae*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* in SHU to cell densities of between $1.1 - 6.7 \times 10^6$ CFU.mL⁻¹. Whole-cell preparations lysed by boiling were used as templates in PCR assays and compared to UPEC preparations. PCR assays were analysed by agarose gel electrophoresis, and only UPEC yielded a DNA PCR product (data not shown).

Mouse urine contains inhibitory substances that decrease qPCR efficiency

The potential influence of qPCR-inhibitory substances in urine was assessed by analysing urine samples from naïve mice in the qPCR assay, and comparing these to pooled urine samples from four healthy human volunteers. The results of qPCR assays based on a standard amount of pGU2470 template and a dilution series of urine that comprised concentrations ranging from 34% to 0.002% urine are shown in Table 2. These assays established an inhibitory effect of mouse urine on the qPCR assay and defined a final maximum reaction percentage of 0.272% mouse urine was suitable to avoid reaction inhibition; at this percentage, the observed C_T value was not significantly different to those of control qPCR reactions that contained no urine.

hlyD qPCR quantitation of UPEC from urines of experimentally infected mice

Mice (n=14) were infected with hemolytic UPEC strain CFT073, and urine was collected at 2h, and at 24h post infection. Each urine sample was used for both culture-based CFU determination, and diluted for use in the qPCR assay. These experiments demonstrated that the qPCR assay successfully detected UPEC in all

fourteen of the urine samples from UPEC-infected mice (Table 3). Samples of urine from control non-infected mice yielded no amplicon in qPCR assays, consistent with these urines being free of cultivable bacteria, as determined by the CFU enumeration method. Additionally, we observed significantly higher numbers of copies of *hlyD* as detected by the qPCR assay, compared to the counts of live-cells using the CFU enumeration method (Figure 3; $P < 0.001$).

Discussion

In this study, we aimed to develop a qPCR assay to quantify UPEC in urine samples. We took an approach of validating the assay using urine samples of both human and mouse origin, given the extensive application of mouse models of UTI, and the measurement of bacteriuria in said models as a surrogate of disease progression and severity in research studies. The primary outcome of this study is the successful development of an efficient qPCR assay based on *hlyD*, a gene that is absent in most commensal *E. coli* but present in most UPEC, and particularly pyelonephritis-causing UPEC strains. A secondary, somewhat unexpected finding of this study, is that mouse urine is significantly more inhibitory to the *hlyD*-targeting qPCR assay compared to human urine. This is potentially due to the higher urine concentrating abilities of mouse kidneys, and the presence of higher urea levels in mouse urine compared to humans, which is a known inhibitor of DNA polymerase (Schrader et al., 2012, Yang and Bankir, 2005). Urea levels may also differ between inbred mouse strains, and therefore may impact the level of qPCR inhibition to some degree (Otto et al., 2016).

The findings of the current study have implications for future research studies that might exploit qPCR assays for the measurement of UPEC in mouse urine; for

example, specific dilution approaches based on the inhibitory effect of mouse urine observed in this study, and a consideration of requirements for particular sensitivity limits would be important. The establishment of a qPCR assay to track UPEC bacteriuria, which exhibits good amplification over a linear range and is superior in terms of sensitivity for detection of total UPEC loads compared to bacteriological culture; for example, the ability of qPCR to detect dead or non-cultivable UPEC as well as DNA fragments from degraded UPEC, as discussed in a prior study on qPCR detection of uropathogens (Lehmann et al., 2011), could be useful for host-pathogen interaction studies as well as studies investigating antibiotic therapy of UTI.

Previous studies reporting the development of qPCR assays for quantitative detection of UPEC from urine of patients with bacteriuria have highlighted the utility of this molecular approach for faster and simpler diagnostic value for UTI compared with traditional urine culture (Hinata et al., 2004). These studies have targeted genes other than those contained within the *hlyCABD* operon, namely universal stress protein (*uspA*) (Hinata, Shirakawa, Okada, Shigemura, Kamidono and Gotoh, 2004). Other more complex approaches, such as a multiplex PCR assay based on O-antigen-specific genes have been used to simultaneously detect many different *E. coli* serogroups (Li et al., 2010). In the present study, we were primarily interested in developing a qPCR assay for research applications, centred on hemolysin-positive UPEC strains, rather than potential clinical applications as in diagnostic use; in addition, we targeted *hlyD* to enable close comparison between our findings with those of a prior study that targeted this gene for detecting UPEC bacteriuria in human urine (Enderle et al., 2014). In a previous study that used *hlyD* as a molecular marker for qPCR detection of UPEC regions of significant homology between the

chosen primer and probe sequences and that of a partially genome-sequenced non-pathogenic commensal gut *E. coli* were reported (Enderle, Miller and Pyles, 2014).

Finally, quantification of UPEC DNA using qPCR of the *gapA* gene in tissue homogenates was used to measure bladder UPEC loads in a recent study (Forsyth et al., 2018). Given our focus on bacteriuria, we excluded any analysis of tissue homogenates.

The limitations of this study include some homologous sequence similarities in *A. actinomycetemcomitans* and *M. haemolytica*; however, these bacteria are anaerobic, and difficult to culture so were not assessed in specificity checks. The qPCR in this study could not be used to differentiate live and dead UPEC, and this may also be considered a limitation. In this regard, the quantitative differences between culture and qPCR observed in this study may reflect the presence of dead or non-cultivable viable cells in the urine (Anderson et al., 2004). The higher estimates of bacterial cell concentration by qPCR compared to culture might also reflect cell division dynamics, whereby separation of the nucleoid into daughter cells could amplify qPCR results from a cell that would nevertheless grow as a single colony on agar. Additionally, different reaction efficiencies between the standard curve and the samples (Brankatschk et al., 2012, Svec et al., 2015) might also effect quantitation differences; however, are common to qPCR, and are similar to other studies (Boutaga et al., 2003, Enderle, Miller and Pyles, 2014).

The correlation between qPCR results and urine culture in this study are consistent with a previous study that reported a good correlation between molecular detection of UPEC and standard plate counts (Hinata, Shirakawa, Okada, Shigemura, Kamidono and Gotoh, 2004). It is also notable that, in interpreting the findings of the

current study related to the significant inhibition of the qPCR by mouse urine, many previous studies on qPCR assays using urine have not included any assessment of qPCR inhibition, as reviewed elsewhere (Green et al., 2009). This makes the results of the current study particularly useful in the context of previous literature focused on the detection of bacterial DNA in urine samples using qPCR. Finally, we note that our findings of partial inhibition by mouse urine are consistent with previously reported findings of partial inhibition of rt-QPCR reactions observed when 5 microlitres of sample volume of unprocessed urine was used (Pang et al., 2008).

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Figure 1: Sequence alignments of *hlyD* and homologs with primer and probe locations. Red lines denote primer and probe locations. Abbreviations: EC: *E. coli* i.e. UPEC; AA: *Aggregatibacter actinomycetemcomitans*; AP: *Actinobacillus pleuropneumoniae*; MV: *Mannheimia varigena*; MH: *Mannheimia haemolytica*; AS: *Actinobacillus suis*; BT: *Bibersteinia trehalosi*. Numbers above and to the right of the alignment indicate the location in base pairs within the gene. The shading of conserved nucleotides within GeneDoc was done in three gradients as follows; 100% identity across all sequences (white text on black), 80% identity (white text on dark grey), and 60% identity (black text on light grey).

Figure 2: Standard curve of *hlyD*-qPCR using the plasmid-borne *hlyD* amplicon in pGU2470. Plasmid from 10^9 to 10^0 copies/reaction was run in triplicate using hydrolysis-probe qPCR. There is an inverse relationship between copy number and C_T value. Standard curve is representative of more than 5 independent qPCR runs.

Figure 3: The comparison of UPEC quantification in mouse urine in CFU/ml and copies/ml. Negative results of the PBS samples were excluded from the data analysis. Results at both time points were taken at random and included in the same group. Data was $\log_{10}$ transformed for statistical analysis. *** $p = 0.001$, Wilcoxon Signed-Rank test

Table 1: Linear quantitative range of qPCR using lysed UPEC. UPEC were analysed by qPCR in 2 separate experiments. CFU/ml are estimates from the highest concentration plated on agar.

qPCR Exp 1 copies/ μ l	qPCR Exp 2 copies/ μ l	Std error copies/ μ l	qPCR average copies/mL	Plate count average CFU/ml
3.0×10^5	6.5×10^5	1.8×10^4	4.7×10^7	6.9×10^6
2.7×10^4	8.3×10^4	2.8×10^4	5.5×10^6	6.9×10^5
2.1×10^3	7.3×10^3	2.6×10^3	4.7×10^5	6.9×10^4
1.4×10^2	6.4×10^2	2.5×10^2	3.7×10^4	6.9×10^3
6.2×10^1	1.1×10^2	2.4×10^1	8.6×10^3	6.9×10^2

Table 2: Titration of urine to assess qPCR inhibitory effects. 10^7 copies of pGU2470 was used in each reaction. *: percentage of mouse urine above which the qPCR was significantly inhibited, **: percentage of human urine above which the qPCR was significantly inhibited, NA: no amplification.

Amount of Urine in Reaction (%)	Average C _T			
	Mouse Exp 1	Mouse Exp 2	Human Exp 1	Human Exp 2
34.00	NA	NA	NA	NA
6.800	NA	NA	18.44	18.29
1.360	24.66	23.81	17.72**	17.36**
0.272*	19.96*	20.00*	17.89	17.82
0.054	20.50	20.27	18.12	17.37
0.011	20.69	20.31	18.36	17.67
0.002	20.83	20.46	17.88	17.91
0.000	20.68	21.75	18.67	18.05

Table 3: Frequency of positive and negative UPEC detections by CFU enumeration and qPCR, using urines from infected or non-infected mice.

Infected mouse urine (n=14)	Urine culture	qPCR	Uninfected mouse urine (n=15)	Urine culture	qPCR
UPEC-positive	14	14	UPEC-positive	0	0
UPEC-negative	0	0	UPEC-negative	15	15

Highlights of the article:

- Use of plasmid-borne *hlyD* (on a pJET1.2 backbone) to establish qPCR sensitivity and linearity of amplification.
- *hlyD* probe-primer combination developed and used to detect and quantify whole cells of UPEC, establishing the superior sensitivity of the qPCR assay compared to a traditional quantification method using standard colony counts.
- Description of results showing that mouse urine contains inhibitory substances that decrease qPCR efficiency.
- Application of *hlyD* qPCR to efficiently detect and quantitate hemolytic UPEC in urine samples from infected mice

* 500 *hlyD_F* * 520 * 540 * 560
 EC_CFT073 : GAATTAATAAATACTACCTGAACGGAAGCCTTCCTGATGAGCCTTATTTTCAGAAATGTATCTGAAGAGGAGTACTCGGTTT : 560
 EC_ATCC_25 : GAATTAATAAATACTACCTGAACGGAAGCCTTCCTGATGAGCCTTATTTTCAGAAATGTATCTGAAGAGGAGTACTCGGTTT : 560
 EC_UM146 : GAATTAATAAATACTACCTGAACGGAAGCCTTCCTGATGAGCCTTATTTTCAGAAATGTATCTGAAGAGGAGTACTCGGTTT : 560
 EC_UTI89 : GAATTAATAAATACTACCTGAACGGAAGCCTTCCTGATGAGCCTTATTTTCAGAAATGTATCTGAAGAGGAGTACTCGGTTT : 560
 EC_536 : GAATTAATAAATACTACCTGAACGGAAGCCTTCCTGATGAGCCTTATTTTCAGAAATGTATCTGAAGAGGAGTACTCGGTTT : 560
 EC_pi1_536 : GAATTAATAAATACTACCTGAACGGAAGCCTTCCTGATGAGCCTTATTTTCAGAAATGTATCTGAAGAGGAGTACTCGGTTT : 560
 EC_pi2_536 : GAATTAATAAATACTTCCTGAACGGAACGCTTCCTGATGAATCTTATTTTCAGAAATGTATCTGAAGAGGAGTACTCGGTTT : 560
 EC_ABU8397 : GAATTAATAAATACTTCCTGAACGGAACGCTTCCTGATGAGCCTTATTTTCAGAAATGTATCTGAAGAGGAGTACTCGGTTT : 560
 AA_D7S-1 : GAAAAAGATCAATATACCAATATAGATTITTTCTAA---AATGATTTTGCCTTTTATGACGGAACGATCABAAAAAGAGT : 557
 AA_HK1651 : GAAAAAGATCAATATACCAATATAGATTITTTCTAA---AATGATTTTGCCTTTTATGACGGAACGATCABAAAAAGAGT : 557
 AA_ANH9381 : GAAAAAGATCAATATACCAATATAGATTITTTCTAA---AATGATTTTGCCTTTTATGACGGAACGATCABAAAAAGAGT : 557
 AP_L20sero : CGCGCGGATAGCTTGCCTCTCATTCGACGACGACAAA---GGATGATTTTAAACATGCTACGGAAGAGATTAAPACCAGAAAT : 557
 AP_AP76 : CGCGCGGATAGCTTGCCTCTCATTCGACGACGACAAA---GGATGATTTTAAACATGCTACGGAAGAGATTAAPACCAGAAAT : 557
 AP_JL03 : GAAACACATGATATACCTTTATGGATTITTAACCA---AGCTGATTTTGCCTTTTATGACGGAAGAGATTAAGACTAACGC : 557
 MV_USDA-AR : GAAAAAGAGTCAATTACCGGTTTATCGATTATCTCAA---CACCGATTTTAAAGATTCATCGGAGAGACAGGTTACGTAT : 557
 MH_USDA-AR : GAAAAAGAGTCCCTTGCCTGATGATTATCTAG---AACCGATTTTAAAGATTCATCGGAGAGAGATCGACTACGTAT : 557
 MH_USMARC : GAAAAAGAGTCCCTTGCCTGATGATTATCTAG---AACCGATTTTAAAGATTCATCGGAGAGAGATCGACTACGTAT : 557
 MH_D174 : GAAAAAGAGTCCCTTGCCTGATGATTATCTAG---AACCGATTTTAAAGATTCATCGGAGAGAGATCGACTACGTAT : 557
 MH_M42548 : GAAAAAGAGTCCCTTGCCTGATGATTATCTAG---AACCGATTTTAAAGATTCATCGGAGAGAGATCGACTACGTAT : 557
 AS_ATCC_33 : GAAACGATCATTTTGCCTATTATCGAATTAACACA---TAATGATCTTAGTCAAGCCTCTGAAGAGGATTAAGACACGGAT : 557
 AS_H91_038 : GAAACGATCATTTTGCCTATTATCGAATTAACACA---TAATGATCTTAGTCAAGCCTCTGAAGAGGATTAAGACACGGAT : 557
 BT_USDA-AR : GAAAAAGAGTCCCTTGCCTGATGATTATCTAG---AACCGATTTTAAAGATTCATCGGAGAGAGATCGACTACGTAT : 557
 Gaa A T CC T A T c A Ttt at c GAaG A a g t

* 580 * *hlyD_P* 600 * 620 * 640
 EC_CFT073 : AACTTCTTTGATAAAAAGACAGTTTTCCTCATGGCAAAAACAGAGTATCABAAAAGAACTGAATCTGGCTAAGAAAAGAG : 640
 EC_ATCC_25 : AACTTCTTTGATAAAAAGACAGTTTTCCTCATGGCAAAAACAGAGTATCABAAAAGAACTGAATCTGGCTAAGAAAAGAG : 640
 EC_UM146 : AACTTCTTTGATAAAAAGACAGTTTTCCTCATGGCAAAAACAGAGTATCABAAAAGAACTGAATCTGGCTAAGAAAAGAG : 640
 EC_UTI89 : AACTTCTTTGATAAAAAGACAGTTTTCCTCATGGCAAAAACAGAGTATCABAAAAGAACTGAATCTGGCTAAGAAAAGAG : 640
 EC_536 : AACTTCTTTGATAAAAAGACAGTTTTCCTCATGGCAAAAACAGAGTATCABAAAAGAACTGAATCTGGCTAAGAAAAGAG : 640
 EC_pi1_536 : AACTTCTTTGATAAAAAGACAGTTTTCCTCATGGCAAAAACAGAGTATCABAAAAGAACTGAATCTGGCTAAGAAAAGAG : 640
 EC_pi2_536 : AACTTCTTTGATAAAAAGACAGTTTTCCTCATGGCAAAAACAGAGTATCABAAAAGAACTGAATCTGGCTAAGAAAAGAG : 640
 EC_ABU8397 : AACTTCTTTGATAAAAAGACAGTTTTCCTCATGGCAAAAACAGAGTATCABAAAAGAACTGAATCTGGCTAAGAAAAGAG : 640
 AA_D7S-1 : GACTTTACTGATGAAGACCAATCTCTCTACTTGGCAAAAACACGCCATCAGAAACCTTGAATCTTTAATAAAAAGAG : 637
 AA_HK1651 : GACTTTACTGATGAAGACCAATCTCTCTACTTGGCAAAAACACGCCATCAGAAACCTTGAATCTTTAATAAAAAGAG : 637
 AA_ANH9381 : GACTTTACTGATGAAGACCAATCTCTCTACTTGGCAAAAACACGCCATCAGAAACCTTGAATCTTTAATAAAAAGAG : 637
 AP_L20sero : TCGCTATTTCATCCAGGCAATTTGAAGCTTGGCAAAAACCAAAATGATCABAAAAGAACTAGCTTTGGACGCTGAGAG : 637
 AP_AP76 : TCGCTATTTCATCCAGGCAATTTGAAGCTTGGCAAAAACCAAAATGATCABAAAAGAACTAGCTTTGGACGCTGAGAG : 637
 AP_JL03 : ACGTCATTTAATATCCGACCAATTTGAGCTTGGCAAAAACCAAAATATCAGAAAGAACTAGCGTATACGCTAAGACAG : 637
 MV_USDA-AR : TAAGCATTTAATCGAAGACCAATACACGCTTGGCAAAAACCAAAATACGCAAAACCTTAGCTTTAATACGCTAAGAGATG : 637
 MH_USDA-AR : TAAACACTTAATTGAGGACCAATACACGCTTGGCAAAAACCAAAATACGCAAAACCTTAGCGTATATAGCGTAAAGAGG : 637
 MH_USMARC : TAAACACTTAATTGAGGACCAATACACGCTTGGCAAAAACCAAAATACGCAAAACCTTAGCGTATATAGCGTAAAGAGG : 637
 MH_D174 : TAAACACTTAATTGAGGACCAATACACGCTTGGCAAAAACCAAAATACGCAAAACCTTAGCGTATATAGCGTAAAGAGG : 637
 MH_M42548 : TAAACACTTAATTGAGGACCAATACACGCTTGGCAAAAACCAAAATACGCAAAACCTTAGCGTATATAGCGTAAAGAGG : 637
 AS_ATCC_33 : TCGTCATTTAATTTGTTGACCAATTCGAACGCTGGCAAAAACCAAAATATCABAAAAGAACTGGTATTACACGCCGAGAG : 637
 AS_H91_038 : TCGTCATTTAATTTGTTGACCAATTCGAACGCTGGCAAAAACCAAAATATCABAAAAGAACTGGTATTACACGCCGAGAG : 637
 BT_USDA-AR : TAAACACTTAATTGAGGACCAATACACGCTTGGCAAAAACCAAAATACGCAAAACCTTAGCGTATATAGCGTAAAGAGG : 637
 tT AT GA CA T ac TGGCAAAA CA aa CA AAa T A aaA G

* *hlyD_R* 660 * 680 * 700 * 720
 EC_CFT073 : CAGAGCGGATTACAAATACCTTGCCTGATAAACCGTTATGAAATTTATCGACAGTTGAAAAAGCCGCTCGGATGATTTT : 720
 EC_ATCC_25 : CAGAGCGGATTACAAATACCTTGCCTGATAAACCGTTATGAAATTTATCGACAGTTGAAAAAGCCGCTCGGATGATTTT : 720
 EC_UM146 : CAGAGCGGATTACAAATACCTTGCCTGATAAACCGTTATGAAATTTATCGACAGTTGAAAAAGCCGCTCGGATGATTTT : 720
 EC_UTI89 : CAGAGCGGATTACAAATACCTTGCCTGATAAACCGTTATGAAATTTATCGACAGTTGAAAAAGCCGCTCGGATGATTTT : 720
 EC_536 : CAGAGCGGATTACAAATACCTTGCCTGATAAACCGTTATGAAATTTATCGACAGTTGAAAAAGCCGCTCGGATGATTTT : 720
 EC_pi1_536 : CAGAGCGGATTACAAATACCTTGCCTGATAAACCGTTATGAAATTTATCGACAGTTGAAAAAGCCGCTCGGATGATTTT : 720
 EC_pi2_536 : CAGAGCGGATTACAAATACCTTGCCTGATAAACCGTTATGAAATTTATCGACAGTTGAAAAAGCCGCTCGGATGATTTT : 720
 EC_ABU8397 : CAGAGCGGATTACAAATACCTTGCCTGATAAACCGTTATGAAATTTATCGACAGTTGAAAAAGCCGCTCGGATGATTTT : 720
 AA_D7S-1 : CGGAAAACACTCGTTATTGGCAGCAATAAAAAATATGAGGGTCTGATTAATACAGAGCAAGTCCGATTTGGATGATTTT : 717
 AA_HK1651 : CGGAAAACACTCGTTATTGGCAGCAATAAAAAATATGAGGGTCTGATTAATACAGAGCAAGTCCGATTTGGATGATTTT : 717
 AA_ANH9381 : CGGAAAACACTCGTTATTGGCAGCAATAAAAAATATGAGGGTCTGATTAATACAGAGCAAGTCCGATTTGGATGATTTT : 717
 AP_L20sero : CAGAAAACCAACAGGTTCTAGCTAATATTCGTAATATGAGGGGATCAGTCAGTTGAAATGAAAGATTAAGAGATCTT : 717
 AP_AP76 : CAGAAAACCAACAGGTTCTAGCTAATATTCGTAATATGAGGGGATCAGTCAGTTGAAATGAAAGATTAAGAGATCTT : 717
 AP_JL03 : CTGAAAACCAACAGATTTAGCAAAATACCGTAATATGAGAGCGCTAGTCTATGAAAGGAGAAATTAAGTGATTTA : 717
 MV_USDA-AR : CTGAAAACCAACAAATATCTGCTTACGTTTCGTAATATGAGGGTGCAACTCTATGAGCAGAGAAAAATTAAGAGACTTT : 717
 MH_USDA-AR : CTGAAAACCAACAAATATTTGCTATGTCCTGTAATATGAGGGTGCAACACCTATTTGAAACAGAAAAATTAAGAGACTTT : 717
 MH_USMARC : CTGAAAACCAACAAATATTTGCTATGTCCTGTAATATGAGGGTGCAACACCTATTTGAAACAGAAAAATTAAGAGACTTT : 717
 MH_D174 : CTGAAAACCAACAAATATTTGCTATGTCCTGTAATATGAGGGTGCAACACCTATTTGAAACAGAAAAATTAAGAGACTTT : 717
 MH_M42548 : CTGAAAACCAACAAATATTTGCTATGTCCTGTAATATGAGGGTGCAACACCTATTTGAAACAGAAAAATTAAGAGACTTT : 717
 AS_ATCC_33 : CTGAGAAAACCAACGGTTTATGCTAATATTCGTAATATGAGGGGATAGCCAGTGAACAGGAAAAATTAAGAGACTTT : 717
 AS_H91_038 : CTGAGAAAACCAACGGTTTATGCTAATATTCGTAATATGAGGGGATAGCCAGTGAACAGGAAAAATTAAGAGACTTT : 717
 BT_USDA-AR : CTGAGAAAACCAACAAATATTTGCTATGTCCTGTAATATGAGGGTGCAACACCTATTTGAAACAGAAAAATTAAGAGACTTT : 717
 C GA A aAc t t GC t T TATGaa g t GAa A T a GA tT

Figure 1

Figure 2

Figure 3

Accepted Manuscript

Quantification of bacteriuria caused by Hemolysin-positive *Escherichia coli* in human and mouse urine using quantitative polymerase chain reaction (qPCR) targeting *hlyD*

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