The Effect of Urine Concentration and pH on the Growth of *Escherichia Coli* in Canine Urine In Vitro

L.A. Thornton, R.K. Burchell [ID], S.E. Burton, N. Lopez-Villalobos, D. Pereira, I. MacEwan, C. Fang, A.C. Hatmodjo, M.A. Nelson, A. Grinberg, N. Velathanthiri, and A. Gal [ID]

Background: Lower urinary tract infections are common in dogs, and *Escherichia coli* is the most common bacterial pathogen isolated. The literature has conflicting evidence regarding the inhibitory effects of urine concentration and pH on *E. coli* growth.

Hypothesis/Objectives: To determine the effect of different pH and urine concentrations on *E. coli* growth in vitro.

Animals: Voided urine samples from 10 apparently healthy spayed female dogs were used.

Methods: A matrix of 9 urine specific gravity (USG; 1.010, 1.020, and 1.030) and pH (5.5, 7.0, and 8.5) combinations was prepared by diluting and titrating filtered voided urine samples. Three *E. coli* isolates were obtained from urine of female dogs with signs of lower urinary tract infection and cultured at different urine pH and USG combinations in wells of a microtiter plate. The number of *E. coli* colony-forming units (CFU) per mL of urine was calculated after aerobic incubation of the urine at 37°C for 18 hours, and statistically compared.

Results: Significant differences were identified in the mean log CFU/mL among different combinations of pH and USG. The lowest log CFU/mL were observed in alkaline concentrated urine (pH 8.5 and USG 1.030).

Conclusions and Clinical Importance: *Escherichia coli* in vitro growth was higher in neutral to acidic and diluted urine compared to alkaline and concentrated urine. The impact of non-alkalizing diluting diets on the incidence of *E. coli* lower urinary tract infections should be further explored.

Key words: Dog; *E. coli*; Osmolality; Lower urinary tract infection.

Lower urinary tract infections (LUTI, including asymptomatic bacteriuria) are common in dogs. The reported prevalence of LUTI in female dogs is approximately 26.6%, and the lifetime risk for LUTI in dogs is 14%.[1,2] The risk of a positive urine culture is 2.5 and 1.5 times higher in spayed and intact female dogs, respectively, than in neutered male dogs,[3] and older spayed females are at the highest risk.[3] Most LUTI are monomicrobial,[4] with *Escherichia coli* being the most commonly isolated bacterial pathogen.[3–9] In dogs and people, the infecting *E. coli* strain is often the host's predominant rectal *E. coli* strain, consistent with the fecal reservoir being the main source from which the bacteria enter the urinary tract.[10] Primary lower urinary tract pathology, nosocomial infections, and immunosuppressive diseases and drugs predispose dogs to LUTI.[9,11,12] When a temporary or permanent breach in the host defense mechanisms occurs, LUTI can develop,[13,14] whereas LUTI is

From the School of Veterinary Sciences, Massey University, Palmerston North, Manawatu-Wanganui, New Zealand (Thornton, Burchell, Burton, Lopez-Villalobos, Pereira, MacEwan, Fang, Hatmodjo, Nelson, Grinberg, Velathanthiri, Gal).

Abbreviations:

USG	urine specific gravity
CFU	colony forming units
LUTI	lower urinary tract infection
NaOH	sodium hydroxide
HCl	hydrogen chloride
PBS	phosphate buffered solution

unlikely to develop in the presence of normal host defense mechanisms.[15,16]

The innate defense mechanisms of the host protect against invasion of bacteria, and the physicochemical properties of urine may be part of this defense system.[17] Specifically, the potential mechanisms underlying antibacterial properties of urine include low pH and high urine concentration.[17] These antibacterial properties have been suggested by many, but remain unproven, and conflicting evidence is found in the literature about the effects of pH and urine concentration on bacterial growth in urine.

In children and in vitro, high urine osmolality inhibits the growth of *E. coli*.[18,19] However, it is the toxic effect of urea in concentrated urine that is a major inhibitor of *E. coli* growth rather than the osmotic effect of urinary organic acids, sodium, and potassium.[19] Some studies however, indicate that urine concentration does not affect *E. coli* growth. For example, urine concentration did not affect the growth of *E. coli* in feline urine,[20] and therefore, other substances in concentrated urine and their interaction with increased urine osmolality possibly contribute to the inhibition of bacterial growth.[21,22]

In people, acidic urine inhibited *E. coli* growth in vitro,[19,23] but in vivo in cats, no correlation was found between pH and bacterial growth.[21] Similarly in dogs with hypercortisolism or diabetes mellitus, neither urine concentration nor pH correlated with the presence of LUTI.[11]

Table 1. Nine combinations of USG and pH used to determine the effect of urine concentration and pH on *E. coli* growth in vitro.

USG 1.010, pH 5.5	USG 1.020, pH 5.5	USG 1.030, pH 5.5
USG 1.010, pH 7.0	USG 1.020, pH 7.0	USG 1.030, pH 7.0
USG 1.010, pH 8.5	USG 1.020, pH 8.5	USG 1.030, pH 8.5

Based on the above findings, a clear cause-and-effect relationship between urine pH and concentration and growth of *E. coli* is lacking, and to our knowledge, no previous studies have assessed it in dogs. We hypothesized that increased urinary pH and concentration would inhibit *E. coli* growth in canine urine in vitro compared to dilute urine with neutral to acidic pH. Our purpose, therefore, was to determine the effect of urine concentration and pH on the growth of *E. coli* in canine urine in vitro.

Materials and Methods

Animals

Dogs consisted of 10 apparently healthy neutered staff-owned female dogs. The median age of the dogs was 6.5 years (range, 2–10 years); 4 were crossbreeds, 2 were Greyhounds and 1 each were Hungarian Vizsla, Maremma sheepdog, Labrador retriever, and Border collie. The inclusion criterion was sex being female neutered; exclusion criteria were antibiotic treatment and the presence of clinical signs consistent with LUTI (eg, hematuria, pollakiuria, dysuria, stranguria) within the preceding 3 weeks. The Massey University's Animal Ethics Committee approved the study protocol (protocol #16/45) and the owners gave informed consent before recruitment of the dogs.

Study Design

After overnight water restriction (>8 hours), 10 morning, voided, mid-stream urine samples (1 from each dog) were collected directly into sterile polypropylene urine collection containers, and the samples were refrigerated at 4°C within 2 hours of collection and for no longer than 4 hours before processing. Each of the collected urine samples was filtered through a 0.22 μm filter,[a] immediately before dilution and titration into 9 different predetermined pH and urine specific gravity (USG) combinations (Table 1) using phosphate-buffered solution (PBS), 0.1 M sodium hydroxide (NaOH),[b] and 0.1 M hydrogen chloride.[b] A pH meter[c] and handheld temperature-compensated refractometer[d] were used to determine urine pH and USG.

Bacterial Isolates

A clinical diagnostic laboratory[e] provided 3 *E. coli* isolates cultured from female dogs with signs of LUTI. Glycerol stocks from each strain were stored at −80°C for use over the entire study period. Before use, the cultures were streaked onto nutrient agar[f] plates from the frozen stock and incubated for 18 hours at 37°C. Suspension of 1–2 colonies of each *E. coli* isolate in 7 mL PBS was adjusted relative to a 0.5 McFarland equivalence turbidity standard.[g]

In Vitro Inoculation

Each of the 3 bacterial isolates was incubated in the titrated and diluted urine samples for 4 hours in wells of a microtiter plate. Briefly, 180 μL of the 9 urine solutions from each dog was dispensed into the top row of 3 separate sterile 96-well plates (1 plate per *E. coli* isolate).

Twenty microliters of an *E. coli* suspension in PBS, of turbidity equivalent to the 0.5 McFarland turbidity standard, was dispensed into each 1 of the 180 μL of urine solutions and incubated at 37°C for 4 hours. Subsequently, serial 10-fold dilutions from 10^{-1} to 10^{-7} were performed and 40 μL of the 10^{-4} to 10^{-7} dilutions was inoculated onto nutrient agar plates. Plates were incubated at 37°C for 18 hours and those plates showing bacterial growth of 30–300 colonies were used to calculate the number of colony-forming units (CFU) per mL of incubated urine. Controls included uninoculated filtered urine (as sterility control) and bacteria diluted in sterile PBS without urine (as positive control).

Statistical Analysis

Data were analyzed using statistical software.[h] The number of *E. coli* CFUs was analyzed using the MIXED procedure with a linear model that included the fixed effect of bacterial isolate, pH, and urine concentration, the interaction among these 3 factors and the inoculated dose of *E. coli* as covariates. Multiple mean comparisons between combinations of pH and USG were performed using the least significant difference test as implemented in the MIXED procedure. Significant difference among the means was set at $P < 0.05$.

Results

The median initial urine pH was 5.84 (range, 5.4–7.18) and median initial USG was 1.047 (range, 1.037-1.065). The number of inoculated bacteria as a covariate did not have a significant effect on the model ($P = 0.07$). Each of the fixed effects, pH, USG, and bacterial strain, had a statistical significant effect on the dependent variable, CFU.

The Effect of Escherichia Coli Strain

The growth of the first and second *E. coli* isolates was significantly higher than that of the third *E. coli* isolate ($P < 0.01$) but no significant difference was found between the first and second isolates ($P = 0.16$).

The Effect of pH
Generally, growth of *E. coli* was higher in acidic compared to alkaline urine (Fig 1).

Differences in pH at USG 1.030
At a USG of 1.030, growth of *E. coli* was significantly higher at pH 5.5 than at pH 8.5 for all 3 isolates (first isolate, $P = 0.007$; second isolate, $P = 0.043$; third isolate, $P < 0.001$).

Also, growth of *E. coli* was significantly higher at neutral pH than at either acidic or alkaline pH. More specifically, *E. coli* growth was significantly lower at pH 5.5 than at pH 7.0 for the first isolate ($P = 0.01$) and for the second isolate ($P = 0.028$), and significantly higher at pH 7.0 than at pH 8.5 for all 3 isolates ($P < 0.001$ for all 3 isolates).

Differences in pH at USG 1.020
At a USG of 1.020, *E. coli* growth was significantly higher at pH 7.0 than at pH 8.5 for the second isolate ($P = 0.048$) and third isolate ($P < 0.001$), and higher at pH 5.5 than at pH 8.5 for the third isolate ($P < 0.001$).

Differences in pH at USG 1.010
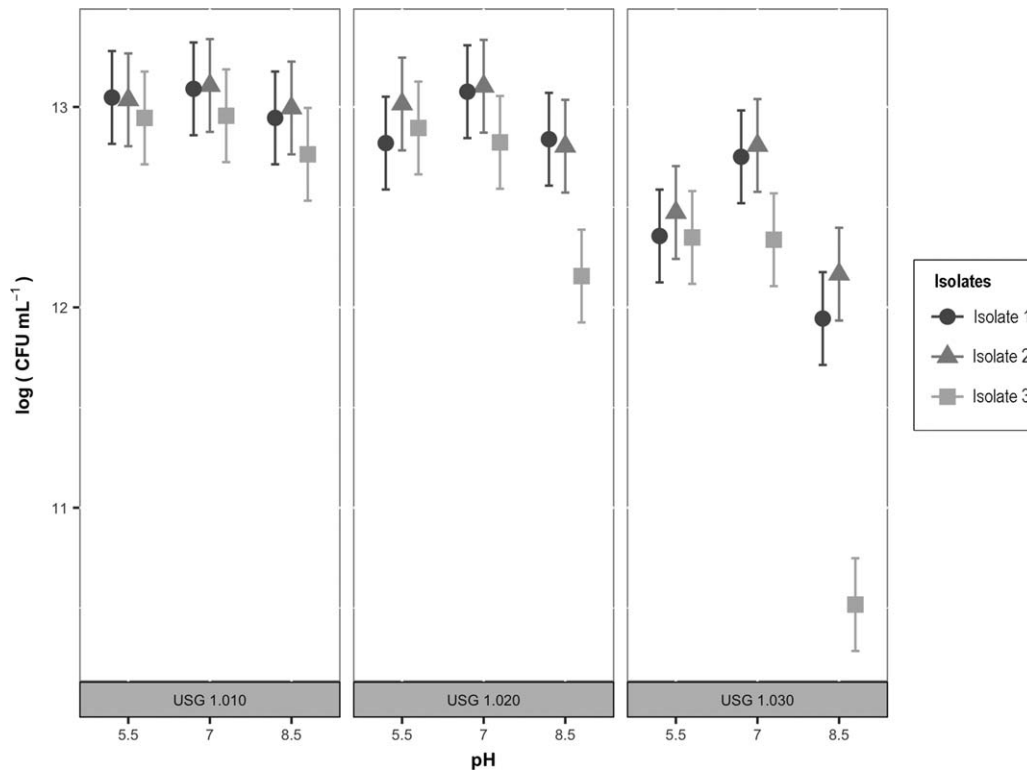At a USG of 1.010, growth of *E. coli* was not significantly different among any of the pHs.

Fig 1. Mean log CFU/mL (±SE) of three *Escherichia coli* isolates plotted against pH and USG. CFU, colony forming unit; SE, standard error; USG, urine specific gravity. See text for information about multiple comparisons.

The Effect of USG

Overall, growth of *E. coli* was higher in diluted as compared to concentrated urine ($P < 0.01$; Fig 1).

Differences in USG at pH 5.5

At a pH of 5.5, growth of *E. coli* was significantly higher at USG 1.010 than at 1.030 for all 3 isolates (first isolate, $P < 0.001$; second isolate, $P = 0.003$; third isolate, $P < 0.001$). Also, growth of *E. coli* was significantly higher at USG 1.020 than at 1.030 for all 3 isolates (first isolate, $P = 0.002$; second isolate, $P < 0.001$; third isolate, $P < 0.001$).

Differences in USG at pH 7.0

At pH 7.0, growth of *E. coli* was significantly higher at USG 1.010 than at 1.030 for all 3 isolates (first isolate, $P = 0.026$; second isolate, $P = 0.049$; third isolate, $P < 0.001$). Also, growth of *E. coli* was significantly higher at USG 1.020 than at 1.030 for the first isolate ($P = 0.033$) and third isolate ($P = 0.002$).

Differences in USG at pH 8.5

At a pH of 8.5, growth of *E. coli* was significantly higher at USG 1.010 than 1.030 for all 3 isolates (first isolate, $P < 0.001$; second isolate, $P < 0.001$; third isolate, $P < 0.001$). Also, growth of *E. coli* was significantly higher at USG 1.010 than at 1.020 for the third isolate ($P < 0.001$). Lastly, growth of *E. coli* was significantly higher at USG 1.020 than at 1.030 for all 3 isolates (first isolate, $P < 0.001$; second isolate, $P < 0.001$; third isolate, $P < 0.001$).

Discussion

We found that *E. coli* grew better in dilute urine than in concentrated urine, and that *E. coli* growth was higher in acidic as compared to alkaline urine but higher at neutral pH (pH 7) than at either acidic or alkaline pH. These results indicate a strong link between urine concentration and pH and growth of *E. coli* in vitro.

Urine contains relatively high concentrations of urea, creatinine, amino acids, organic acids, inorganic ions (eg, ammonia, sodium, potassium), purines, and pyrimidines, which could affect *E. coli* growth.[24] Increasing osmolality with sodium chloride or ammonium were not found to increase antibacterial activity.[19] Antibacterial activity also was not affected by decreasing osmolality when urea concentration remained the same. Conversely, decreasing urea concentration but preserving ammonia concentration and osmolality decreased antibacterial activity,[18] and increasing urea concentration markedly increased the antibacterial activity of urine.[19] Therefore, urea concentration may be a more important determinant of antibacterial activity than osmolality or ammonia concentration. Further work is needed to investigate whether increasing osmolality limits bacterial growth, or whether the effect is related to the particular solute that is causing the increase in osmolality. Also, several other factors must be considered when assessing the effect of urine concentration on the growth of *E. coli* in vivo. For example, alterations in osmolality are likely to affect host defenses as well as bacteria. Furthermore, diluting urine also increases the frequency of voiding and complete emptying of the bladder.[25]

We found that *E. coli* growth was higher in dilute urine as compared to concentrated urine. Our results are similar to those of an in vivo study in children that found a significant correlation between increasing urine concentration and inhibition of bacterial growth,[20] but different from results of a study in cats and dogs in which decreasing urine concentration was not associated with risk of a positive urine culture, independent of disease status.[11,26] Possible reasons for these discrepancies are inherent differences between in vivo and in vitro study designs, differences among *E. coli* strains with regard to osmotolerance,[27] and species differences in the content of urinary osmoprotective substances. For example, in 1 in vitro study,[28] osmotically stressed *E. coli* were able to grow in hyperosmotic urine by utilization of urinary glycine and proline betaine as osmoprotective molecules. In that study, differences were found in urinary glycine and proline betaine concentrations among species resulting in differences in growth thresholds in a hyperosmotic environment.

We found that although *E. coli* growth was highest at neutral pH, acidic urine allowed for a higher growth than did alkaline urine. Our results are in agreement with previous studies in people that showed that in vitro growth was impaired at pH < 5.5 and > 7.6,[29] and in vivo work that found no significant differences between *E. coli* growth in urine of pH 6.5 and 7.4, but a higher growth rate at pH 5.5 than 5.0.[30] Our results do however contrast with an in vivo study in cats in which no association was found between urine pH and the presence of a positive culture, independent of disease,[26] and in an in vivo study in dogs with hypercortisolism, diabetes mellitus or both, in which no association was found between the presence of a positive urine culture and urine pH.[11] Possible explanations include interspecies differences and inherent differences between in vitro and in vivo studies. For example, in an in vitro study, the pH is kept constant whereas in vivo dynamic changes in urine pH occur and as a result pH is not kept constant.

Diet influences urine composition including urinary pH and urine concentration.[31,32] Healthy dogs produce slightly acidic urine when being fed most diets, including adult maintenance diets.[31] Some diets (eg, Prescription Diet Canine c/d,[i]) are formulated to further acidify the urine. Most diets designed for other conditions (eg, those designed to manage fiber-responsive diseases and to aid in management of chronic kidney disease) do not substantially alter the pH of urine from that associated with adult maintenance diets (ie, which resulted in a slightly acidic urine pH). For all urolith mineral types, except infection-induced struvite calculi, feeding high-moisture diets (>75% moisture) and diluting the urine, aiming for USG <1.020, are the cornerstones of prevention.[33,34] Only a few diets are designed to avoid acidic urine pH and these include those designed to prevent calcium oxalate (urine pH >6.5), urate (pH >8.0), and cysteine (pH >7.5) urolithiasis.[33] Given that medical dietary intervention is common in dogs, our results suggest that non-alkalinizing diets that dilute the urine might provide optimal conditions for *E. coli* growth. Hence, our study provides grounds for a future prospective clinical investigation of whether conditions or interventions leading to non-alkaline dilute urine increase the risk of *E. coli* LUTI.

We designed our study to specifically assess the direct effect of urine concentration and pH on *E. coli* growth in vitro. Hence, we did not take into account the possible effects that urine pH and concentration may have on different components of the innate immune system and the physiologic mechanism of micturition. The incubation of *E. coli* was performed under aerobic conditions and the lower oxygen tension in urine in vivo might affect *E. coli* growth differently. We used USG to measure urinary concentration rather than osmolality, but a strong linear correlation exists between USG and osmolality within a physiological range in dogs.[35] We used NaOH to alkalinize the urine, and we cannot exclude the possibility that the high concentration of sodium may have suppressed growth. High extracellular concentrations of sodium can inhibit *E. coli* growth,[36] but this effect is more marked under anaerobic conditions.[36] The possible effect of sodium could be addressed in a future study by using potassium hydroxide instead of NaOH as an alkalinizing agent.

In conclusion, non-alkaline dilute urine from healthy female dogs promoted in vitro growth of *E. coli* isolated from dogs with LUTI. Future epidemiological studies should determine if an increased incidence of LUTI with *E. coli* occurs in dogs that consume non-alkalinizing diluting diets. Similarly, we suggest further investigation of the effect of urine pH and concentration on *E. coli* growth in an in vivo system. Meanwhile, in accordance with the results of our study, caution is advised against a general recommendation for non-alkalinizing and diluting urinary interventions, especially in dogs with comorbid or recurrent *E. coli* LUTI.

Footnotes

Author Contributions

Acknowledgments

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