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Intravesical compartments in the bladder can affect microbiological culturing outcome: An experimental study in pigs

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Urinary tract infections (UTIs) are one of the most common bacterial infections worldwide. The diagnosis is mainly dependent on the quantitative identification of uropathogens in urine from suspected patients and therefore, it is essential that bacterial count in urine specimens accurately reflects the bacterial population inside the bladder. In this study, we collected the first- and last urine specimens during a single full voiding from pigs with experimentally induced UTI and compared the urine density and bacterial counts of the collected specimens. We found that specimens from the same voiding varied in density and in bacterial counts by up to a thousand-fold with significant impact on diagnostic sensitivity. In conclusion, the porcine bladder can simultaneously contain intravesical compartments of varying density and bacterial counts that greatly influences microbiological analysis. The distribution of bacteria in intravesical compartments is critical to consider when using pigs as biomedical models of UTI. Furthermore, the similarities of porcine and human anatomy and physiology, suggest that the results are likely to be translatable to human, in which case potential UTIs could be overlooked by today's diagnostic procedures.

KEYWORDS

urinary tract infection (UTI), uropathogenic escherichia coli (UPEC), urinary bladder, pig, swine, large animal model, urine dipstick test

1 Introduction

Urinary tract infection (UTI) is a common bacterial infection and a leading cause of urosepsis (1, 2). The main parameter for diagnosing UTI is the identification and quantification of uropathogens in urine specimens from patients with relevant symptoms (3). The interpretation of urinary culture results relies on a quantitative approach where specific thresholds of bacteriuria, often 10^5 bacteria·mL^{-1} is considered significant and compatible with clinical disease (3). Consequently, patients presenting urinary bacterial counts below these thresholds will often not be offered antibiotic treatment according to most international guidelines (3). It is therefore essential, that urinary specimens accurately represent the bacterial population inside the bladder.

During experimental work with a new model of UTI in pigs, we have observed that the density of the first urine specimen collected by a transurethral bladder catheter can deviate significantly from the last urine specimen collected during the same voiding suggesting that urinary compartments of varying density may exist inside the bladder (unpublished observations) (4–7). Uropathogenic *Escherichia coli* (UPEC) are known to occupy different niches in the urinary tract, and even locally in the bladder UPEC is present in phenotypically different planktonic and sessile subpopulations (8). Significantly different composition of urine samples during the same voiding could indicate the presence of different niches in the bladder urine, which may not only result in different local concentrations of bacteria but also bacterial subpopulations of different phenotype (9). To investigate if bladder urine compartments may support bacterial subpopulations, we designed an experimental study in pigs where the first- and last urine specimens from a single voiding was collected and compared to assess the prevalence- and impact on microbiological culturing during UTI.

2 Method

2.1 Animals

We used forty-seven female pigs (Danish Landrace x Yorkshire) from a local vendor with highest health status according to the Danish SPF system (10). The pigs were allocated to three sub-studies summarized in a flowchart (Figure 1). Six pigs were used for study I (cystoscopy analysis), 22 pigs for study II (the infection experiments) of which 3 were euthanized of reasons unrelated to the infection, and 19 pigs for study III (diagnostic sensitivity). Pigs used for cystoscopy weighted approximately 45 to 50 kg and pigs used for infection weighted 41.7 to 75.5 kg. The animals were group housed in standard $3m^2$ enclosures with sawdust bedding and enrichment in the form of various toys, music and daily human interaction. Pigs were given standard feed and free access to water and were inspected at least twice daily by trained animal technicians. A veterinarian was always available.

2.2 Cystoscopy

Pigs were placed in supine position on the operating bed and cystoscopy performed using a rigid ureteroscope 27002 K (Karl

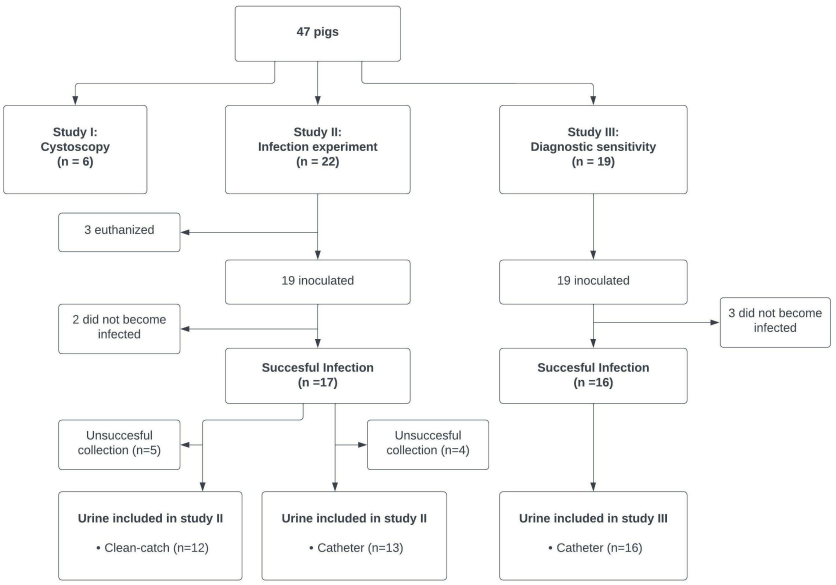

FIGURE 1
Flowchart showing the allocation of pigs in the three sub-studies.

Storz). The bladder was drained for 100-150 mL until approximately 50 mL urine was left in the bladder (estimated by the operator). Hereafter, 180 mL saline was instilled to distend the bladder and allow for clear visualization of the mucosa. Images were recorded using a tele pack vet X LED (Karl Storz).

2.3 Bacteria

We used two common model organisms of uropathogenic *Escherichia coli*; 36 animals were infected with the human cystitis strain UTI89 and two animals with the human pyelonephritis strain CFT073 (11, 12). Both strains were pre-incubated in lysogeny broth (LB) to optimize type-1 fimbrial expression (5). On the day of the infection, the broth was centrifuged at 10,000 x g for 20 minutes and the pellet resuspended in saline and adjusted to an optical density of 1.0 at 600 nm corresponding to $1{\cdot}10^9$ colony forming units (CFU)·mL^{-1}. Further dilutions were made in saline to reach 10^2·CFU·mL^{-1} (UTI89) or 10^6·CFU·mL^{-1} (CFT073).

2.4 Pig infection model

The infection model is based on Stærk et al. (5) In short, pigs were placed in supine position and catheterized using a Charrière 10 Foley type catheter (Rüsch) or Charrière 12 bladder catheter (SpeediCath, Coloplast) depending on the size of the animal. The first 5 mL were discarded to clear the catheter for potential contaminants and hereafter 10 mL urine was collected, thus representing midstream specimens similar to human sampling practice. Hereafter, the bladder was drained until the flow seized, indicating that the bladder was mostly empty, and then the catheter was repositioned slightly to remove remaining residual urine which was collected in another 15 mL tube. For study III, the exact placement of the catheter was more rigorously controlled by advancing the catheter all the way into the bladder. After discarding approximately 5 mL, the first urine specimen was collected. When the flow seized, the catheter was slowly withdrawn resulting in further urine output from the bottom compartment, which was collected as the last urine specimen. The same operator performed all catheterization in study III.

To infect the animals, an inoculum of 100 mL was instilled into the empty bladder after which the catheter was clamped for 1 hour and then emptied to ensure equal basis of infection. After incubation for 24 hours (UTI89) or 6 hours (CFT073), the pigs were catheterized again to collect pairs of urine specimens as described above. A useful pair of urine samples, i.e., first and last urine specimens from a single voiding, was successfully collected from 29 infected pigs (five animals did not develop infection and from four animals, a representative pair of urine was not successfully collected because the animals spontaneously urinated during sedation). Clean-catch urine was collected during spontaneous micturition using an approach described previously (13). In short, after cleaning the pens as part of the morning maintenance routine, the animals were hustled towards their designated dunging site which stimulates micturition. Urine was collected in 100 mL sterile cups by clean-catch after 2-3 seconds to clear the urethra for contaminants (first urine specimen) and again when the flow started to slow (last urine specimen).

The golden standard for positive infection in the pigs was a urine monoculture of *E. coli* and bladder inflammation (determined by gross pathology of removed whole bladders).

2.5 Anesthesia and euthanization

For collecting urine samples where only short term anesthesia was needed, pigs were premedicated with an intramuscular injection of medetomidine (Cepetor 0.05 $mg{\cdot}kg^{-1}$), butorphanol (Butomidor 0.2 $mg{\cdot}kg^{-1}$) and midazolam (Midazolam 0.2 $mg{\cdot}kg^{-1}$).To reverse the alpha-2 adrenergic effect of medetomidine, an intramuscular injection of atipamezole (Antisedan 0.12 $mg{\cdot}kg^{-1}$) was administered as soon as the pig had been returned to the pen.

For bacterial inoculation, pigs were premedicated as described above. After placing an intravenous access in the ear vein, propofol was administered intravenously as a constant rate infusion, to maintain a sufficient level of sedation while conserving spontaneous respiration. To prevent hypoxia, oxygen was administered *via* a face mask and pulse oximetry and respiration pattern monitored continuously. Atipamezole (Antisedan 0.12 $mg{\cdot}kg^{-1}$) was administered as soon as the pig had been administered propofol.

For cystoscopy, pigs were premedicated with an intramuscular injection of medetomidine 0.03 $mg{\cdot}kg^{-1}$, midazolam 0.25 $mg{\cdot}kg^{-1}$, ketamine 5 $mg{\cdot}kg^{-1}$ and butorphanol 0.2 $mg{\cdot}kg^{-1}$ and an IV access placed, while monitoring oxygen saturation with pulse oximetry. General anaesthesia (GA) was induced with propofol, an orotracheal tube was placed and anaesthesia was maintained with propofol 10 mg/kg/h and fentanyl 20 $\mu g{\cdot}kg^{-1}{\cdot}h^{-1}$ while achieving normoventilation mechanically. While in GA, non-invasive blood pressure, electrocardiogram, heart rate, oxygen saturation and capnography were continuously monitored. All pigs were euthanized with 5 ml i.v. pentobarbital (200 $mg{\cdot}mL^{-1}$).

2.6 Microbiological culturing and urinalysis

Urine specimens were plated in serial dilutions on LB agar (SSI Diagnostica) and incubated ON at 35°C. Plate colonies were

quantified by manual counting and selected single colonies from each plate were verified to be *E. coli* using matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) using a Microflex LT instrument (Bruker Daltonics), Flex control software and the MALDI Biotyper 4.1.90 software (Bruker Daltonics). Urine collected from infected pigs by catheter or clean-catch are not burdened by contamination as previously demonstrated (13); this was also the case in this study in which contaminants were negligible (most often below the detection limit), i.e., urine cultures were mostly monocultures of *E. coli* and occasional contaminants were easily excluded from colony counts. Urine specific gravity (equals density, hereafter denoted USG) was measured in specimens at room temperature using a digital refractometer (UG-α, Atago). Urine specimens were subjected to urine dipstick test (Combur 7 Test, Roche). The results were interpreted by two individuals, and in the case of discrepancy, the test was re-evaluated until agreement on the result was reached. Dipstick score was calculated by adding the score of each parameter: glucose (0-4), ketones (0-3), leucocytes (0-3), nitrite (0-1), protein (0-3) and blood (0-4).

2.7 Ethical approval

All urine specimens used in this study was collected as part of tandem project performed in the same series of pigs from three studies approved by the Danish Animal Experimentation Inspectorate, license number 2021-15-0201-00931; 2021-15-0201-00821; and 2021-15-0201-00814.

2.8 Statistics and calculations

Statistical analyses were performed using GraphPad Prism version 9.3.1. Comparisons of first- and last urine specimens were done using nonparametric Wilcoxon matched-paired signed rank test. Contingency analyses were performed using Fisher's exact test. Calculations were performed with Microsoft Excel version 2203. USG ratio was calculated as $\frac{(USG_1-1)}{(USG_2-1)}$ and CFU ratios as $\frac{CFU_1}{CFU_2}$, where CFU1 was always the specimen with the highest CFU.

3 Results

3.1 Study I: Cystoscopy

3.1.1 High- and low-density urine segregates in the bladder *in vivo*

To visualize how urine may segregate inside the bladder, we inserted a cystoscope in six pigs and emptied the bladder through the working channel until approximately 50 mL urine was left (estimated by the operator). Hereafter, 180 mL of saline was instilled to distend the bladder and allow for clear visualization of the mucosa. The saline and residual urine was distinctly segregated visualized by a clear line when the cystoscope was placed in the transitional phase (Figures 2A, B). Urine specimens collected from each fluid phase showed a clear difference in density, indicated by a change in color (Figures 2D, E). This demonstrates that residual urine and saline, the latter which has a lower density than urine, does not spontaneously mix inside the bladder despite that saline was instilled relatively forcefully through the cystoscope working channel. In two pigs, this segregation was visibly evident before instillation of saline, demonstrating that urine may segregate naturally in intravesical compartments (Figure 2C). The cystoscopy and gross pathology of removed whole bladders showed normal tissue structure, with no signs of bladder diverticula, vesicoureteral reflux or other abnormalities (data not shown).

3.2 Study II: Infection study

3.2.1 Urine density varies between first- and last urine specimens in healthy pigs

To assess the variation in urine density in pigs, first- and last urine specimens from a single voiding, were collected from nine control pigs (i.e., before subjected to experimental infection) using a transurethral bladder catheter. The ratio of USG between first- and last urine specimens is summarized in Table 1. In three of nine animals, the USG values were notably different between first and last specimens with a 1.7 to 2.3-fold change in USG. This is a considerable difference considering the narrow range of normal USG with a maximum theoretical fold-change of approximately 6 in both pigs and humans.

3.2.2 Intravesical compartments contains different bacterial concentrations

To assess the influence of intravesical compartments on urinary bacterial counts during UTI, pairs of urine samples were collected through bladder catheters in 13 pigs with experimentally induced UTI. USG was only measured in 10 of the samples with a considerable difference in USG (1.9 to 4.1) in four of the 10 infected pigs (Table 1). In these pigs, urine specimens from each compartment were clearly different in color (Figure 2F). Bacterial counts where quantified in all urine samples of which five of the 13 animals had CFU-ratios above 30 and one animal with a ratio of 769 (Table 1). In only one animal was the CFU-ratio 1, indicating a uniform distribution of bacteria inside the bladder.

3.2.3 Bacterial distribution in compartments is recapitulated in urine during spontaneous micturition

The segregation of dilute- and concentrated urine observed in the animals could potentially be intensified by a diuretic

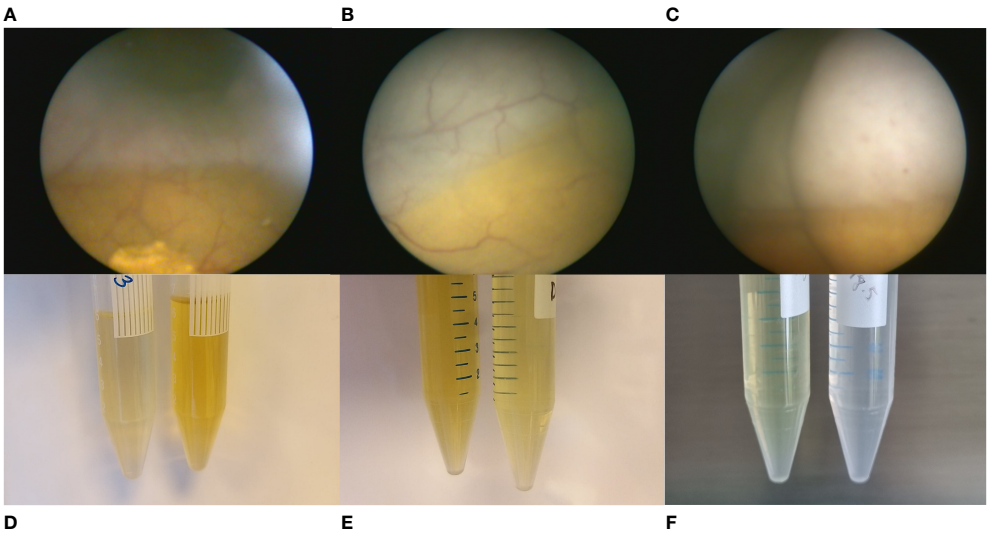

FIGURE 2
A cystoscope was inserted into the bladder of 6 pigs and 100-150 mL urine removed until roughly 50-100 mL was left in the bladder (estimated by the operator). Hereafter, 180 mL saline was instilled into the bladder through the cystoscope working channel and subsequent imaging showed distinct segregation of saline and urine: the latter always at the bottom due to higher density **(A, B)**. In two pigs, the segregation of urine was observed before saline was instilled **(C)**. Urine- and saline specimens collected from each fluid phase by the cystoscope working channel was visibly different in density **(D)**. The variation in density was also observed in most pairs of urine, (i.e. first- and last urine specimens) collected by transurethral bladder catheter from pigs with ongoing UTI **(E, F)**.

response induced by the a2-adrenergic effect of medetomidine, an anesthetic drug used to sedate the animals for cystoscopy and bladder-catheterization. Moreover, urine segregation might only form in the resting, sedated animals, which are also placed in supine position to facilitate catheterization and cystoscopy thus potentially shifting the placement of the urinary comparts. To assess if this was the case, we collected clean-catch urine specimens from the pigs during spontaneous micturition. The CFU-ratio was 1.0 in 42% of animals (5 of 12) when urine was collected by clean-catch compared to only 8% of animals (1 of 12) when urine was collected by bladder catheter during sedation. However, the CFU-ratio was considerably different in most of the urines collected by clean-catch (7 of 12) and in one animal a CFU-ratio of 195 was detected (Table 1).

3.3 Study III: Diagnostic sensitivity

3.3.1 Culture- and dipstick test of last urine specimens have increased diagnostic sensitivity

In most urine pairs collected by bladder catheter in study II, the specimen of highest density was the urine collected first (n=9), but in other animals (n=4) the last specimen was of higher density. Furthermore, the highest CFU count was not always detected in the urine specimens of highest density. These differences may be explained by the exact placement of the catheter during voiding, which was not systematically controlled for: hence, if the catheter was inserted deeper into the bladder, the first specimen may represent urine from the back or upper compartment containing the urine of lesser density and vice versa. To assess the influence of the exact placement of the catheter, we performed a separate experiment in which the placement of the catheter was rigorously controlled for. To do so, the same operator catheterized all animals, and the catheter was pushed all the way in before urine was collected (after discarding the first 5-10 mL). When the flow stopped, the catheter was slowly pulled out resulting in further urine output that could be collected, thus representing the last urine specimen.Sixteen animals with experimental UTI was used for this experiment. From 14 of the 16 animals, the last urine specimen collected by this approach contained the highest bacterial counts, up to 1091-fold higher than the first specimen (pig no. 36) (Table 2). The urine density was highest in the first urine specimen in 15 of 16 animals (Table 2).

Furthermore, to assess the potential clinical impact of urinary compartments on the reliability of diagnosing UTI, we compared dipstick test score, and colony counts of each specimen to the standard cut-off threshold of significant bacteriuria in human patients ($>10^5$ CFU·mL^{-1}). In 13 of 16 pigs with confirmed UTI (determined by monoculture of *E. coli* and positive bladder inflammation), colony counts above 10^5 CFU·mL^{-1} were detected in the last urine specimen, corresponding with clinical disease. However, when analyzing the first urine specimen only 6 of 16 pigs showed colony counts above 10^5 CFU·mL^{-1}. This difference in diagnostic sensitivity was statistically significant (p=0.029, Fisher's exact test). Dipstick test score also differed

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TABLE 1 Urine sample analysis.

Control			
ID	Fold-change, USG	Fold change, CFU (CFU·mL^{-1})	Collection method
1	1.7	NA	Catheter
2	1.9	NA	Catheter
3	1.0	NA	Catheter
4	1.3	NA	Catheter
5	1.0	NA	Catheter
6	1.2	NA	Catheter
7	1.1	NA	Catheter
8	1.0	NA	Catheter
9	2.3	NA	Catheter
Infection			
10	4.1	170	Catheter
11	0.4	89	Catheter
12	1.8	3	Catheter
13	1.1	50	Catheter
14	0.4	4	Catheter
15	1.9	3	Catheter
16	2.5	3	Catheter
17	1.2	1	Catheter
18	0.7	4	Catheter
19	1.0	769	Catheter
20	NA	31	Catheter
21	NA	2	Catheter
22	NA	5	Catheter
23	1.0	3	Clean-catch
24	1.0	11	Clean-catch
25	1.0	1	Clean-catch
26	1.0	1	Clean-catch
27	1.0	195	Clean-catch
28	1.0	2	Clean-catch
29	1.3	1	Clean-catch
30	0.8	3	Clean-catch
31	0.9	2	Clean-catch
32	1.0	1	Clean-catch
33	1.0	1	Clean-catch
34	2.0	4	Clean-catch

USG, urine specific gravity; CFU, colony forming units. NA, Not applicable.

significantly (p=0.0059) between first urine specimens (mean 4.9, SD: 2.7) and last urine specimens (mean: 6.9, SD: 2.7) (Figure 3). Glucose and ketones were never positive. Last urine specimens typically contained cellular debris in contrast to first urine specimens which were mainly clear.

4 Discussion

In this experimental porcine study, we show that urine inside the bladder segregate in compartments, that may vary considerably in terms of urine density and bacterial counts. Consequently, we show that the sensitivity to detect significant bacteriuria (>10^5 CFU·$mL^{-1)}$ is significantly reduced when culturing first- urine specimens compared to last urine specimens.

In most cases, independent of sampling method, a difference in CFU between first- and last urine specimens was also associated with a difference in USG, but this was not always the case. In three of the animals, where some of the highest differences in CFU was observed (no. 12, no. 18 and no. 26), the USG ratio was 1.1, 1.0 and 1.0, respectively, suggesting that other factors of urine, besides density, may influence bacterial distribution inside the bladder. This was also supported by the observation, that in many animals the highest CFU was detected in the most dilute specimen. When the placement of the catheter was rigorously controlled for, we found that the last urine specimen was most often of lower density (15 of 16) but contained higher colony counts (14 of 16). The last urine specimen is likely to represent a small volume of residual urine that is not successfully voided during spontaneous micturition, but can be collected by catheter, thus explaining the higher bacterial counts of these specimens. Furthermore, the higher colony counts in last urine specimens may be explained by the increased content of sediments, i.e., exfoliated cells that may be significantly covered in adherent bacteria and possibly early-stage biofilm. This is supported by the observation, that last urine specimens often contained visible sediments. The significance of intravesical compartments was also witnessed by the significant difference in dipstick test results between first- and last urine specimens. Particularly leucocyte detection was significantly increased in last urine specimens, which most likely, is also a result of increased sediments in these samples.

Urine density and colony counts tended to be more homogenous in urine collected by clean-catch suggesting that (i) the diuretic side-effect of medetomidine may interfere, and perhaps intensify, intravesical compartments or (ii) that the potential intravesical compartments are mixed during the intricate contraction of the detrusor muscle associated with natural micturition. One limitation to urine specimens collected by clean-catch, which could potentially interfere with the result, is that the first urine specimen is near-impossible to collect because it is difficult to anticipate when the pig will micturate spontaneously. Furthermore, first-voided urine specimens may be misleading or difficult to interpret, in terms of microbiological culturing, as the sample is more likely to be contaminated with

TABLE 2 Impact of urinary compartments on diagnostic threshold of significant bacteriuria.

ID	Fold-change, USG	Fold change, CFU (CFU·mL^{-1})	CFU >10^5 ·mL^{-1} (First urine)	CFU >10^5 ·mL^{-1} (Last urine)
35	1.6	115	–	+
36	1.5	1091	–	+
37	3.7	6	–	–
38	1.6	0.2	+	+
39	1.1	0.6	+	+
40	1.7	2	–	–
41	2.1	24	+	+
42	3.7	95	+	+
43	2.3	1.4	+	+
44	2.6	375	–	+
45	0.5	34	–	+
46	1.8	375	–	+
47	4.2	133	–	+
48	3.2	6	–	–
49	1.7	15	–	+
50	1.8	1.3	+	+

"+": Growth >10^5 CFU/mL; "-": Growth <10^5 CFU/mL.

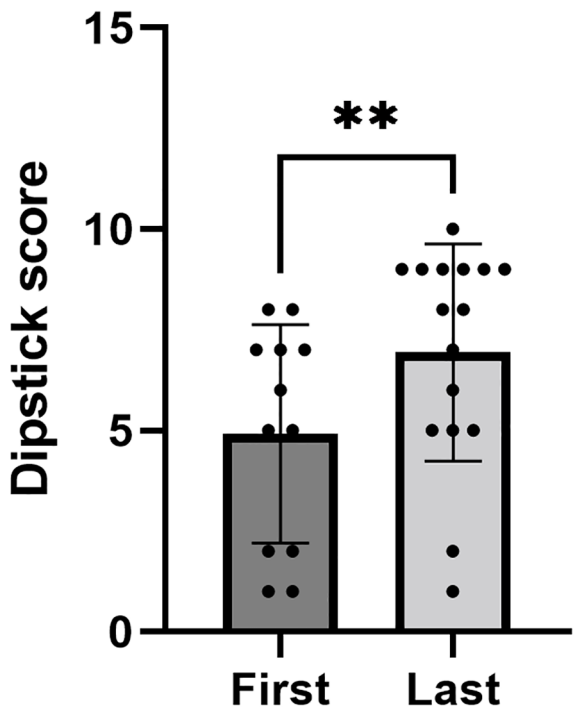

FIGURE 3
Dipstick test was performed on first- and last urine specimens collected from pigs (n=16) experimentally infected with uropathogenic *Escherichia coli*. The dipstick test score was significantly higher in the last urine specimen. **p=0.0059, Wilcoxon matched-pairs signed rank test.

commensals from the urogenital microbiota compared to a mid-stream catch. Therefore, clean-catch urine specimens were collected after 2-3 seconds of voiding – to clear contaminants in the urethra - and thus, reflects a midstream urine sample.

4.1 Clinical significance

From a perspective of translating these finding to humans, it is important to recognize the high resemblance of the porcine urinary tract and kidneys to that of humans (14, 15). The renal anatomy and physiology of pigs is more similar to humans than even primates (16–18). Consequently, the urine production and range of urine concentration is essentially the same, suggesting that the density-dependent distribution of urine compartments, as observed in this study, is likely to be shared between these two species (19, 20). Urinary bladder anatomy and function is also highly similar between pigs and humans (16).

Besides the anatomical and physiological similarities, the pig is also highlighted as an excellent animal model of human UTI because pigs are natural host of human uropathogens and highly susceptible to UTI which occurs naturally in this animal (19). Overall, the parameters regarding host and pathogen suggest that these results from pigs are translatable to humans. If so, one puzzling feature of clinical observations can be explained: previous studies have shown that some patients presenting with symptoms of UTI, had coliform-colony counts of as low as 10 or 10^2 CFU·mL^{-1} in midstream urine specimens (21). Such patients could represent cases where urine specimens were collected from an intravesical compartment of lower bacterial concentration resulting in urine culture results below the significant bacterial threshold. Furthermore, the influence of intravesical compartments on urine dipstick test results demonstrated in this study, may partly explain the low reliability of these tests in clinical practice (22).

Further studies are needed to confirm if intravesical compartments with varying bacterial counts are present in human patients: most likely are patients that are immobile, or patients in which a rapid shift from concentrated to dilute urine production is induced, i.e., patients who is administered diuretic drugs or iv fluids - the latter which is a standard first-line medical prescription to suspected UTI patients admitted to the emergency department (23). If similar conditions are true for humans, potential UTIs could be overlooked by today's diagnostic procedures. Based on this study, clean-catch midstream urine specimens from patients with spontaneous micturition may be less influenced by potential intravesical compartments, as the compartments are likely to be mixed during detrusor activity associated with natural micturition.

In conclusion, we show that intravesical compartments is common in the urinary bladder of pigs, and as a result, that bacterial counts and dipstick test results can significantly vary between these compartments. While this study adds new information about the intravesical distribution of urine and bacteria in large animals, the implication in human UTI needs to be investigated in further studies.

Data availability statement

The datasets presented in this article are not readily available because The raw dataset supporting this study cannot be made public available at the time of submission due to confidentiality agreements with involved funders. Requests to access the datasets should be directed to kristian.staerk@rsyd.dk.

Ethics statement

The animal study was reviewed and approved by The Danish Animal Experimentation Inspectorate.

Author contributions

KS: Conceptualization, investigation, methodology, project administration, writing – original draft, writing – review and editing. LL: Resources, writing – review and editing. LN: Investigation, writing – review and editing. TA: Funding acquisition, methodology, supervision,

writing – review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author LN is employed at Coloplast A/S.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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