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The infectious capacity of *Enterococcus faecalis*, *Staphylococcus aureus*, and *Staphylococcus saprophyticus* in a porcine model of urinary tract infection

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The purpose of this study was to establish a porcine model of urinary tract infection (UTI) with gram-positive uropathogens. Ten female domestic pigs were experimentally inoculated with human UTI isolates of *Enterococcus faecalis* (n = 3), *Staphylococcus saprophyticus* (n = 3), or S*taphylococcus aureus* (n = 4) and followed with regular urine samples. Bladders and kidneys were aseptically removed at termination (5–7 days post infection) and assessed by gross pathology and bacterial enumeration. *Enterococcus faecalis* (n = 3 of 3) and *S. aureus* (n = 2 of 4) successfully colonized the pig bladders. Inoculation with *S. saprophyticus* never resulted in detectable bacteriuria. All infected pigs had cleared the infection spontaneously before termination. Surprisingly, three (of four) pigs inoculated with *S. aureus* led to spontaneous infection with opportunistic pathogens. Also, one pig colonized with *E. faecalis* resulted in spontaneous infection with *E. coli*. In conlusion, the pig supports experimental UTI with *E. faecalis* for up to 24 h but not prolonged infection. *S. aureus* and *S. saprophyticus* fails to cause UTI in pigs and other animals should be considered for studying these pathogens.

Key words: Urinary tract infection; pig; large animal model; *Enterococcus faecalis*; *Staphylococcus aureus*; *Staphylococcus saprophyticus*.

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Urinary tract infection (UTI) is one of the most common bacterial infections worldwide [1]. Uncomplicated UTIs, that is, in otherwise healthy individuals, are mainly caused by uropathogenic *Escherichia coli* responsible for up to 90% of cases [2]. In urologic patients who often have considerable risk factors for UTI such as compromised bladder emptying, urolithiasis, tumors, or catheters, the variety of etiological agents is more nuanced often involving gram-positive microorganism, *Pseudomonas* spp., or yeasts [2]. There is a significant focus on *E. coli* UTI in basic and translational research which leaves gram-positive-associated UTI relatively understudied, despite the significance of these pathogens in urologic patients [3]. Particularly, the increase in vancomycin-resistant enterococci (VRE) threatens to reduce future treatment options and warrants the development of novel antibacterial modalities against UTI caused by gram-positive bacteria [4, 5].

Animal models are critical for deciphering basic pathogenesis in infectious diseases, for assessing antibiotic efficacy and for drug safety testing.

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However, to be able to truthfully extrapolate results from animals to humans, the animals must as accurately as possibly reflect the various aspects of the disease in humans. For UTI, the mouse is the go-to model, but fails to recapitulate human UTI in several fundamental aspects [3, 6, 7]. Most importantly, the high osmolarity of mouse urine possess bacteriostatic properties which is a potential confounder when studying pathogenesis and drug efficacy in this animal [8].

Recently, pigs have demonstrated great potential as animal model for studying human UTI and catheter-associated UTI, as the infection in this animal shares important characteristics with the human disease [7, 9–13]. Porcine models of lower and ascending *E. coli* UTI are well described, but no porcine studies have investigated uropathogenic microorganism other than *E. coli*. The objective of the present study was to investigate the infectious capacity of three important gram-positive uropathogens (*Enterococcus faecalis*, *Staphyloccoccus saprophyticus*, and S*taphylococcus aureus*) in pigs with the overall aim of establishing a porcine UTI model of gram-positive etiology.

MATERIALS AND METHODS

Animals

Ten 13-week-old female domestic pigs (Landrace × Yorkshire) were sourced from a local supplier (Kokkenborg Aps, Stenstrup, Denmark) and allowed 7 days acclimatization before the experiments began. The animals were housed in communal enclosures (3 m^2 per animal) with sawdust bedding. They were fed a standard diet with free access to water. The experiments were approved by the Danish Animal Experiments Inspectorate (license no. 2022-15-0201-01303). The animals were part of a tandem project with another primary focus; hence, on the day of termination, the animals were subjected to a pharmacokinetic study. The pharmacokinetic study was always initiated after the last urine sample was collected to avoid interfering with urinary tract parameters being studied.

Bacteria

The bacteria used in this study has been described previously and are summarized in Table 1 [14]. The *S. aureus* and *E. faecalis* isolates used were isolated from human patients with verified urosepsis, that is, the pathogens were identified in both urine specimens and blood cultures. *S. saprophyticus* was isolated from a urine monoculture as bacteriemia with this pathogen is rare. All isolates were found in the routine diagnostic laboratory of the Department of Clinical Microbiology, Odense University Hospital. To prepare the inoculum, bacteria were grown in brain heart infusion (BHI) medium overnight and centrifuged at 5000 *g* for 5 min to pellet the bacteria. The supernatant was removed, and the pellet resuspended in saline and adjusted to an optical density = 1.0 at 600 nm.

Table 1. Bacterial strains

Strain	Description	References
Staphylococcus aureus 869359	Isolated in urine- and blood culture from a male Parkinson's disease patient with indwelling suprapubic catheter. Methicillin sensitive	Agergaard et al. (2023)
Staphylococcus saprophyticus USs2022	Isolated from urine culture of a cystitis patient	Agergaard et al. (2023)
Enteroccocus faecalis BEf2022	Isolated in urine- and blood culture from a male patient admitted with urosepsis. Ampicillin and vancomycin sensitive	Agergaard et al. (2023)

This suspension was diluted 1000-fold to yield final inoculum concentrations of 10^6 CFU/mL.

Anesthesia

Pigs were premedicated with an intramuscular injection of medetomidine 0.05 mg/kg (Cepetor Vet, ScanVet Animal Health A/S), butorphanol 0.2 mg/kg (Butomidor Vet., Salfarm Danmark), and midazolam 0.2 mg/kg (Midazolam Hameln, hameln pharma gmbh). When complete muscle relaxation was reached, the animals were moved to the operating bed while monitoring oxygen saturation with pulse oximetry. General anesthesia (GA) was induced with propofol and maintained with propofol 10 mg/kg/h (Proposure, ScanVet Animal Health A/S).

Urinary tract infection protocol

The protocol was based on the study conducted by Stærk et al. [9]. Pigs were placed in dorsal recumbency on the operating bed. The urogenital area was washed and disinfected using two rounds of 0.5% chlorhexidine (Meda A/S) and catheterized transurethral using a 10-Fr Foley type catheter (Rüsch) using an approach previously described [15]. The bladder was emptied completely to collect urine and to ensure equal basis of infection. The inoculum (100 mL) was instilled into the bladder and the catheter clamped. After 1 h, the bladder was emptied and the catheter removed.

At termination, sedated animals were placed in dorsal recumbency and the abdomen washed and disinfected. Hereafter, the animals were euthanized with 0.35 mL/kg i.v. pentobarbital (400 mg/mL, Exagon Vet., Salfarm Denmark) and the bladder and kidneys removed aseptically within 10 min postmortem. The organs were immediately transferred to a sterile air-flow cabinet. The bladder was opened by a midline incision between the ureters and washed with saline. The splayed bladders were photographed for gross pathology assessment (see below). Five specimens were punched from the bladder using a drive punch (Ø = 10 mm) and sonicated in 5 mL saline for 5 min at 40 kHz to release tissue-bound bacteria. Aliquots were plated on agar for bacterial enumeration (see below).

Kidneys were bisected through the renal pelvis and one half subsequently sonicated in 15 mL saline and aliquots plated.

During follow-up, urine samples were collected by clean-catch using a previously described technique [16].

The experiments were performed in three rounds of two to four pigs, and the animals in each group were tested in two different rounds to minimize potential bias.

Urine analysis

Urine and tissue aliquots were plated in serial dilutions on CHROMID CPS Elite agar plates (Biomérieux, Ballerup, Denmark) at 35°C in atmospheric air and occasionally on 5% blood agar (SSI diagnostica, Hillerød, Denmark) in atmospheric air or 5% CO_2. Plate colonies were quantified by counting. Bacterial colonies were identified using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) using a Microflex LT instrument (Bruker Daltonics, Bremen, Germany), Flex control software, and the MALDI Biotyper 4.1.90 software (Bruker Daltonics). Urine specific gravity (USG) was measured using a refractometer (UG-α, Atago, Fukaya, Saitama, Japan).

Pathological analysis

Tissues were evaluated by a pathologist blinded to group allocations. The specimens were assessed for edema on a scale from 0 (none to little) to 1 (moderate to strong) and hemorrhaging on a scale from 0 to 3. The overall pathology score was determined by coining the score of the two parameters. Catheter-associated lesions were not considered in the score as they are mechanical focal lesions not associated with the bacterial outcome.

RESULTS

Infectious outcome

Colonization outcome is summarized in Table 2. No detectable bacteria were identified in urine specimens at baseline and all pigs produced urine of USG within the natural range of pigs (Table 2) indicating equal basis of infection. *E. faecalis* successfully colonized all animals (n = 3); however, in two animals (#1 and #2), the bacteria were spontaneously cleared by Day 3 and in the third animal (#3) by Day 7. Pig #1 and #2 showed macroscopic signs of bladder inflammation characterized by hemorrhage (Fig. 1). No inflammatory signs were observed in pig #3. Colonies of *E. faecalis* were undetected in kidneys. Two (of four) animals (#4 and #6) inoculated with *S. aureus*, resisted colonization, determined by no detectable *S. aureus* 24 h post infection. In the other two pigs (#5 and #7), *S. aureus* was detectable in significant numbers at 24 h post infection but cleared by Day 3 (#7) and day 5 (#5). None of the animals inoculated with *S. saprophyticus* (#8–#10) developed bacteriuria and bladders from these animals were characterized by low to no inflammatory lesions.

Table 2. Infectious outcome

No.	Infectious agent	Urine colony-forming units mL^{-1} (identified species)							Pathology score	Average urine-specific gravity (SD)	Study round
		Day 0	Day 1	Day 3	Day 4	Day 5	Day 6	Day 7			
1	*E. faecalis*	<LOD	$>10^5$ (*E. faecalis*)	<LOD	<LOD	<LOD	–	–	2	1.021 (0.008)	1
2	*E. faecalis*	<LOD	$>10^7$ (*E. faecalis*)	<LOD	<LOD	<LOD	–	$>10^8$ (*E. coli*)	1	1.017 (0.008)	1
3	*E. faecalis*	<LOD	$>10^6$ (*E. faecalis*)	$>10^7$ (*E. faecalis*)	–	–	$>10^3$ (*E. faecalis*)	<LOD	0	1.016 (0.004)	3
4	*S. aureus*	<LOD	<LOD	–	<LOD	–	–	<LOD	1	1.013 (0.002)	2
5	*S. aureus*	<LOD	$>10^7$ (*S. aureus*)	$>10^3$ (*S. aureus*) $>10^7$ (*S. suis*)	$>10^5$ (*S. aureus*) $>10^7$ (*S. suis*)	–	–	$>10^7$ (*S. suis*)	1	1.013 (0.002)	2
6	*S. aureus*	<LOD	<LOD	<LOD	$>10^2$ (*P. mairii*)[1]	–	–	–	3	1.020 (0.008)	3
7	*S. aureus*	<LOD	$>10^4$ (*S. aureus*)	<LOD	<LOD	<LOD	$>10^6$ (*P. mairii*)	$>10^6$ (*P. mairii*)	4	1.019 (0.004)	3
8	*S. saprophyticus*	<LOD	<LOD	<LOD	<LOD	–	–	–	1	1.014 (0.004)	2
9	*S. saprophyticus*	<LOD	<LOD	<LOD	<LOD	–	–	<LOD	0	1.013 (0.002)	2
10	*S. saprophyticus*	<LOD	<LOD	<LOD	<LOD	–	–	–	0	1.010 (0.001)	3

LOD, limit of detection (10 CFU/mL).
[1]Urine from catheter replated after 24 h on 5% blood agar in 5% CO_2 incubator.

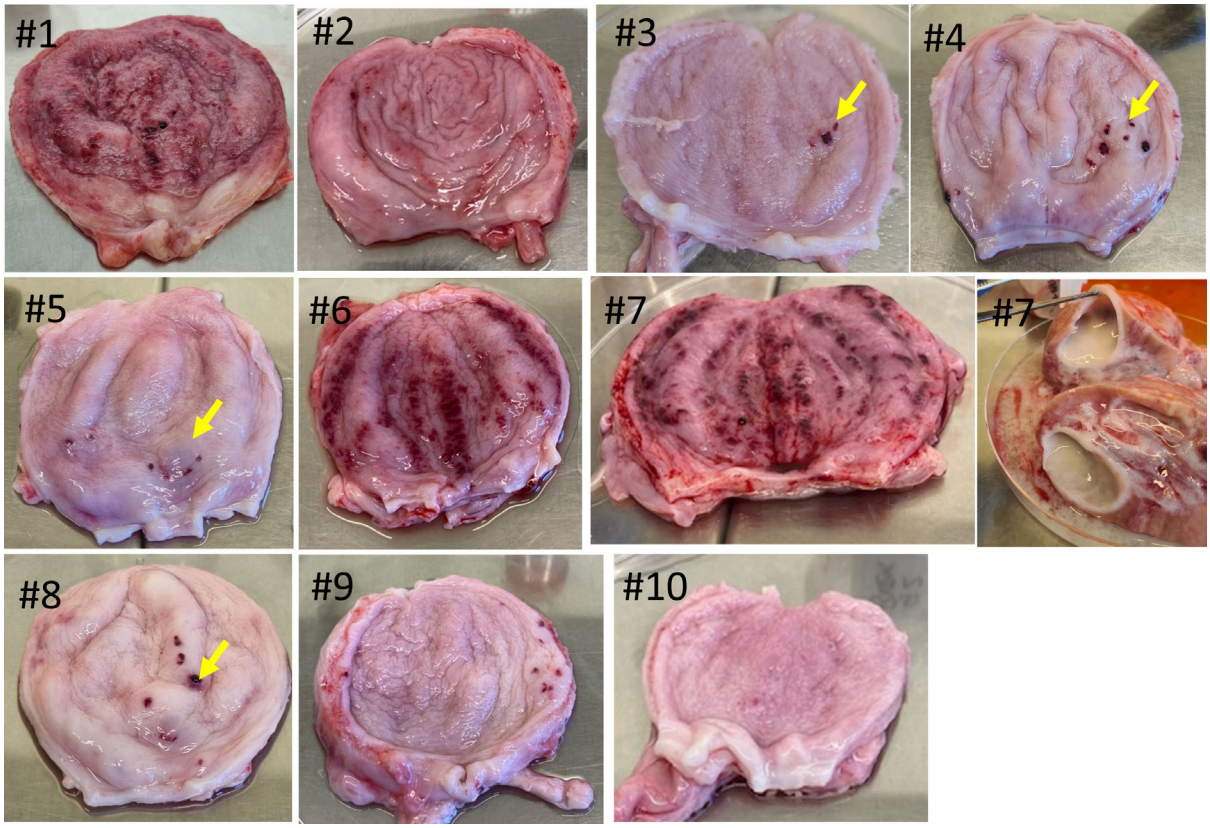

Fig. 1. Gross pathology of whole bladders from #1 to #10. In pig #7, a large kidney abscess was identified along with two minor abscesses. All animals has pinpoint lesions caused by the catheter (most evident on the non-inflamed bladders, yellow arrows).

Pig #6 and #7 (both from the *S. aureus* group) developed spontaneous superinfection with *Pasteurella mairii*, a gram-negative porcine-specific pathogen. *P. mairii* was identified in urine specimens from both animals and also recovered from the bladder and left kidney, including two renal kidney abscesses, from pig #7 (Fig. 1). Gross pathology examination of bladders from #6 and #7 with *P. mairii* bacteriuria revealed distinct signs of inflammation characterized by widespread hemorrhaging (Fig. 1).

Furthermore, pig #5, which was initially infected with *S. aureus* developed bacteriuria with *Streptococcus suis*, culturable in urine specimens from Day 3 and onward and recovered from bladder tissue ($>10^5$ CFU/cm^2), but not kidneys. Bladder examination revealed edema but no signs of hemorrhaging suggesting that the presence of *S. suis* could have been a case of subclinical colonization. Opportunistic infection was also identified in pig no. 2 (initially colonized with *E. faecalis*) from which significant *E. coli* CFU were recovered from urine ($>10^8$ CFU/mL) and bladder surface ($>10^5$ CFU/cm^2). All bladders had focal pinpoint lesions caused by the catheter as previously demonstrated [17].

DISCUSSION

In this study, we evaluated selected uropathogens as infectious agents in a porcine model of UTI. We found that the tested strains (*E. faecalis*, *S. aureus*, and *S. saprophyticus*) were generally poor colonizers of the healthy porcine bladder resulting in short-term colonization or no colonization at all. Surprisingly, we found that inoculation with gram-positive bacteria significantly disposed to opportunistic infection.

Inoculation with *E. faecalis* resulted in bladder colonization of all animals, but in one animal (#3) no signs of inflammation was visible in the bladder indicating that the colonization could have been subclinical (asymptomatic). As all animal remained colonized for 24 h, this pathogen would be relevant to use for short-term challenge studies. This would reflect the typical approach in murine models of

E. faecalis, where the mice are typically terminated within 24 h [18–23]. Although short-term infection may be used for some experimental designs such as vaccine challenge studies, it does not allow for drug efficacy testing as control animals would clear the infection spontaneously. For this type of study, a persistent infection is mandatory as most UTI treatments span across 5–7 days.

S. aureus only resulted in bacteriuria in two of four animals and in one of these (#5) no inflammatory signs were identified suggestive of subclinical colonization. *S. saprophyticus* never gave rise to bacteriuria. Taken together, the study demonstrates that these two pathogens are not reliable for use in porcine UTI models. The inability of *S. saprophyticus* to colonize the porcine bladder sharply contrast infection models in rodents, where *S. saprophyticus* consistently establishes robust infection of the bladder and kidneys in mice and rats for up to 14 days [24, 25]. While this shows that the pig has a specific natural resilience toward UTI with gram-positive pathogens, it may reflect a lower susceptibility also known in humans, in which mainly elderly, urological patients or catheter users are susceptible to these pathogens. Hence, the vague infectious outcome in the present study may be explained by the fact that the pigs were healthy animals with uncompromised bladder function [2].

The poor ability of gram-positive bacteria to colonize healthy bladders contrasts previous pig studies with *E. coli*, a pathogen that at very low inocula triggers significant inflammation and persists in the porcine bladder for weeks upon experimental infection [9, 11, 26]. This suggests that *E. coli* is uniquely adapted for survival in the urinary tract of naturally susceptible larger mammals such as pigs and humans and may explain why this pathogen is the dominant etiological agent in human UTI [2]. Furthermore, we used a high inoculum dose for the gram-positive bacterial isolates that were 10 000-fold higher compared to previous studies with *E. coli*, thus further demonstrating the superiority of *E. coli* to establish and thrive in the urinary tract compared to gram-positive bacteria.

Although this study is limited by the low sample size (relative to rodent studies), the results demonstrates convincingly that gram-positive bacteria are relatively poor colonizers of the bladder. We used only one isolate of each species so it cannot be ruled out that other isolates, which may harbor additional virulence genes, may be more infectious. Similarly, given that all species were human isolates, potential species-specific differences in pathogenesis could influence on the course of the disease in pigs. Another limitation of our study is that the animals were subjected to a pharmacokinetic study on the day of termination. The pharmacokinetic study was always initiated after the last urine specimen to avoid interfering with the bacterial CFU counts in urine specimens. However, the enumeration of bacteria associated with bladder and kidney tissue may potentially have been affected. This was considered in the conclusions drawn from the study.

The occurrence of several opportunistic infections was an unexpected finding in the present study. From our earlier studies in more than 200 pigs experimentally inoculated with *E. coli*, we have never observed opportunistic infections during follow-up. One explanation may be that potential mucosal damage caused by catheterization predisposed the pigs to infection by other microbes in the absence of significant bacteriuria. In the studies with *E. coli* it is likely that the inoculated *E. coli* achieved a high enough density to outcompete the other species that entered the urinary tract, while the gram-positives did not. We have performed experiments before using the same procedure (and thus inducing the same potential trauma), using virulence-deficient *E. coli* mutants [9], *Pseudomonas* spp. [11], and asymptomatic *E. coli* strains [27] that all were unable to colonize the bladder of healthy pigs without causing spontaneous infection with other microbes. Taken together, these experiences suggest that the opportunistic infections did not occur as a result of the catheterization procedure, but rather as a result of the presence of gram-positive bacteria. Infectious synergy between different species of uropathogenic bacteria have been reported in mouse models, where simultaneous co-inoculation increases the risk of ascending UTI (*S. saprophyticus* and *Proteus mirabilis*) or increases the bacterial burden of the urinary tract (*E. coli* and *P. mirabilis*) [28, 29]. Furthermore, group B streptococci have been suggested to suppress innate immune responses during acute UTI, potentially making a more hospitable environment for other bacteria [30]. Common to previous studies on polymicrobial infection is, however, the simultaneous inoculation of both species. To our knowledge, spontaneous and delayed opportunistic infection following mono-inoculation, as seen in the present study, has not been reported. The spontaneous infections are likely facilitated by the pig's natural susceptibility to UTI. Taken together, our study shows that inoculation with gram-positive bacteria dispose to opportunistic infections; however, the limited number of animals in this study is only enough to suggest said hypothesis. The exact mechanism explaining the gram-positive bacterial disposure to opportunistic infection needs further studies with more animals.

CONCLUSIONS

The low susceptibility of pigs to UTI with gram-positive bacteria reflects the low susceptibility in healthy humans. As experimental model, pigs may be used for short term infection experiments using *E. faecalis*. However, for studies involving prolonged follow-up, such as antibiotic treatment experiments, the tested gram-positive isolates are not suitable due to unreliable infection rate and rapid spontaneous clearing.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

FUNDING

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ETHICAL APPROVAL

The experiments were approved by the Danish Animal Experiments Inspectorate (license no. 2022-15-0201-01303) and performed according to the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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