

# *Escherichia coli* type-1 fimbriae are critical to overcome initial bottlenecks of infection upon low-dose inoculation in a porcine model of cystitis

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## Abstract

Most uropathogenic *Escherichia coli* (UPEC) express type-1 fimbriae (T1F), a key virulence factor for urinary tract infection (UTI) in mice. Evidence that conclusively associates this pilus with uropathogenesis in humans has, however, been difficult to obtain. We used an experimental porcine model of cystitis to assess the role of T1F in larger mammals more closely related to humans. Thirty-one pigs were infected with UPEC strain UTI89 or its T1F deficient mutant, UTI89 $\Delta$ *fimH*, at inoculum titres of 10<sup>2</sup> to 10<sup>8</sup> colony forming units per millilitre. Urine and blood samples were collected and analysed 7 and 14 days post-inoculation, and whole bladders were removed at day 14 and analysed for uroepithelium-associated UPEC. All animals were consistently infected and reached high urine titres independent of inoculum titre. UTI89 $\Delta$ *fimH* successfully colonized the bladders of 1/6 pigs compared to 6/6 for the wild-type strain. Intracellular UPEC were detectable in low numbers in whole bladder explants. In conclusion, low doses of UPEC are able to establish robust infections in pigs, similar to what is presumed in humans. T1F are critical for UPEC to surpass initial bottlenecks during infection but may be dispensable once infection is established. While supporting the conclusions from mice studies regarding a general importance of T1F in successfully infecting the host, the porcine UTI models' natural high, more human-like, susceptibility to infection, allowed us to demonstrate a pivotal role of T1F in initial establishment of infection upon a realistic low-inoculum introduction of UPEC in the bladder.

## INTRODUCTION

Urinary tract infection (UTI) is one of the most frequent nosocomial and community-acquired infectious diseases [1, 2]. The most common etiological agent is uropathogenic *Escherichia coli* (UPEC) accounting for 80% of all cases [3]. UPEC is adapted for survival in the urinary tract and uses a range of adhesive fimbriae to resist the hydrodynamic forces of urine flow. The most well-described of these fimbriae is the type-1 fimbria (T1F) which is found in most UPEC strains [4–8]. T1F are hair-like structures that mainly consists of multiple FimA subunits with a FimH tip adhesin that

specifically binds to mannosylated uroplakin receptors on bladder epithelial cells (BECs) [9].

The adhesive properties of T1F are well-documented in cell-culture based assays, and UPEC strains lacking T1F or the FimH tip adhesin are significantly attenuated in murine cystitis models [7, 10, 11]. In these models, T1F not only facilitate adhesion to BECs, but they also induce invasion and establishment of intracellular bacterial colonies [6, 12–14]. Although the experimental evidence suggests a critical role for T1F during human cystitis, it has been difficult to extrapolate results from cell cultures and murine models to human

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**Keywords:** cystitis; pig model; type-1 fimbriae; urinary tract infection; uropathogenic *Escherichia coli*.

**Abbreviations:** BEC, bladder epithelial cell; CFU, colony forming units; DMEM, Dulbecco's modified Eagle medium; Dpi, days post-infection; LB, lysogeny broth; LOD, limit of detection; PBS, phosphate-buffered saline; T1F, type-1 fimbriae; UPEC, uropathogenic *Escherichia coli*; USG, urine specific gravity; UTI, urinary tract infection; VR, void restriction.

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UTI and convincing evidence is still lacking to support the existence and putative role in humans, of the type-1 pilus induced invasion of BEC's observed in mice [7]. Moreover, in an analysis of UPEC in the urine from women with cystitis, most isolates did not express T1F, whereas the same isolates up-regulated T1F expression several hundred-fold during experimental UTI in mice [15]. Recent *in vitro* modelling of bladder epithelium infection has offered some explanation to the discrepancies by showing that planktonic bacteria growing in human urine express little or no T1F whereas bladder epithelial cell-associated bacteria are highly fimbriated [4, 5]. This adds further complexity to the role of T1F and justifies the need for experimental studies in intermediate models that more accurately reflect human disease and in particular the onset of infection in humans, which can bridge the extensive results gathered over the years in cell cultures and mice to humans.

Domestic pigs have been highlighted as an excellent animal model for studying infectious diseases since they more accurately reflect human anatomy, genetics, and physiology, and results from experiments in pigs are considered to be more predictive of human outcome compared to rodent models [16–19]. Furthermore, like humans, many domestic animals including pigs are natural hosts of UPEC and related pathogens [20]. The mouse, on the other hand, is not a natural UPEC host and has historically been challenged by the fact that mice are intrinsically resistant to UTI [17]. Thus, for consistent colonization, the typical infectious dose in murine models is  $10^7$ – $10^9$  c.f.u. ml<sup>-1</sup> which is likely far higher than the titres present during the onset of a natural UTI in humans [21, 22]. Even in studies of non-human primates, high inoculum ( $10^7$ – $10^9$  c.f.u. ml<sup>-1</sup>) have been used to induce infections [23]. Inoculation with excessive bacterial doses is likely to mask key early-stage mechanisms that are critical during the onset of infection and hence, a low-dose UTI model is warranted to shed further light on UTI pathogenesis.

Here, we compared the minimal infectious dose of a reference UPEC strain, UTI89, to a T1F deficient mutant in an optimized porcine challenge model and investigated their respective urine and tissue colonization [24].

## METHODS

### Bacterial strains and media

We used the standard cystitis strain UTI89 originally isolated from a patient with UTI and used in numerous studies [4, 21]. The bacteria were preincubated similar to Hung *et al.* to ensure optimal T1F expression [21]. In short, 25 ml of Lysogeny Broth (LB) was inoculated with a single plate colony and incubated overnight without shaking. The next day, 25 µl of suspension was transferred to a new tubes with 25 ml LB. On the day of infection, the bacterial inoculum was prepared immediately before inoculation by centrifuging the suspension at 2500 g for 20 min, resuspending the pellet in saline and adjusting to OD<sub>600</sub> = 1.0 yielding approximately 10<sup>9</sup> c.f.u. ml<sup>-1</sup>. This suspension was serial diluted until the desired inoculum concentration was achieved (10<sup>8</sup>–10<sup>2</sup> c.f.u. ml<sup>-1</sup>). Low-level

T1F expression was achieved by preincubating UTI89 as described above in sterile filtered porcine urine, pooled from four donors. As a negative control of T1F expression, we used a UTI89 mutant in which the gene encoding for the functional adhesin FimH was deleted (UTI89Δ*fimH*) [25].

### Animals

We used 31 female domestic pigs (Landrace x Yorkshire, mix) of 40 kg (15–16 weeks old) from a pig herd with the highest Danish health status [26]. The animals were acclimatized for 7 days before initiation of experiments and kept together in standard hay-covered pig enclosures of 3 m<sup>2</sup> per animal during the experiments.

### Porcine cystitis model

The porcine cystitis model is based on Nielsen *et al.* [24]. Pigs were catheterized with a *charrière* 10 catheter (Rüsch) followed by urine sample collection and bladder emptying before transurethrally inoculating the animals with 100 ml bacterial suspension. Hereafter, the pigs were subjected to 1 h (VR) by clamping the catheter, unless otherwise stated. After VR, the bladder was emptied, and the catheter removed. Urine samples were collected through a sterile bladder catheter during sedation, and blood samples were collected from the jugular vein. Pigs were euthanized with 5 ml of pentobarbital (200 mg ml<sup>-1</sup>) and bladders aseptically removed within 10 min.

For sedation, pigs were pre-medicated with medetomidine (Cepetor 0.12 mg kg<sup>-1</sup>), butorphanol (Butomidor 0.2 mg kg<sup>-1</sup>) and Midazolam (Midazolam 0.1 mg kg<sup>-1</sup>). Anaesthesia was induced and maintained on propofol after which the pigs were given IM antisedan (0.2 mg kg<sup>-1</sup>) to reverse the α<sub>2</sub>-adrenergic effects of medetomidine.

### Sample analysis

Urine samples were analysed for urine specific gravity (USG) and serially diluted for plating on LB agar (SSI Diagnostica) to quantify the level of bacteriuria. Urine samples from day one were cultured to make sure that no animals were colonized prior to inoculation. Blood samples were centrifuged at 2500 g for 10 min, and plasma was collected and stored at –80 °C. Plasma samples were analysed for anti-UTI89 IgG as described below.

### Whole-cell ELISA

Microtitreplates were coated with 100 µl per well of 1 µg ml<sup>-1</sup> poly-L-lysine (Sigma) for 2 h. Plates were washed and 100 µl bacterial suspension of UTI89 adjusted to OD<sub>600</sub> = 0.2 was added to each well. Plates were then centrifuged at 900 g for 20 min. The bacteria were fixed by adding 0.2% formaldehyde for 20 min. The plates were then washed and blocked with 10% foetal bovine serum-PBS for 1 h. After removing the blocking buffer wells were loaded with 100 µl of two-fold serial dilutions of standards (made by pooled porcine plasma from nine infected pigs and assigned a value of 1000 mU ml<sup>-1</sup>), starting at 1:50 dilution and samples diluted 1:1000 in PBST

in duplicated were incubated for 2 h. Plates were washed and incubated with 0.2 ng per well of secondary rabbit anti-pig-HRP IgG (Invitrogen) for 1 h. After washing, the plates were developed with OPD (Sigma) for 10 min. Absorbance was recorded at 490 nm in a plate reader (Molecular Devices).

### Gentamicin protection assays

The gentamicin protection assays was based on the approach from murine models [27]. In short, whole bladders were immediately transferred to a laminar airflow cabinet, opened by cutting between the ureters and washed in PBS (SSI Diagnostica) to remove non-adherent bacteria. Hereafter, round-shaped specimens ( $\varnothing=10$  mm) were punched from the bladders and three samples were left untreated and three samples were incubated in gentamicin  $300 \mu\text{g ml}^{-1}$  in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum for 1.5 h to kill extracellular bacteria. After washes with saline, all samples were homogenized using a rod disperser at 22000 r.p.m. (IKA Ultra-Turrax T25), and the homogenate was serially diluted and plated on LB agar to quantify intracellular bacteria.

### Yeast cell agglutination

The phenotypical presence of functional T1F was assessed by their ability to agglutinate yeast (*Saccharomyces cerevisiae*) [28]. Bacteria were washed twice in PBS and adjusted to  $\text{OD}_{600}=0.5$ . From this suspension,  $50 \mu\text{l}$  of bacteria were mixed 1:1 with 1% (w/v) yeast suspension in PBS on black disposable reaction cards (Thermo Scientific).

### Statistical analysis

Statistical analyses were performed with GraphPad Prism version 8.0.2. Comparisons between more than two groups were performed with one-way ANOVA with Brown-Forsythe test for variance and Tukey's multiple comparisons test. Comparisons between categorical data was performed with Fischer's exact test.

## RESULTS

### Persistent, high bacterial titre UTI is established in pigs regardless of inoculation dose

Eighteen pigs were divided into four groups (4–6 pigs in each group) and inoculated with 100 ml of UPEC suspension in concentrations of  $10^8$ ,  $10^6$ ,  $10^4$ , and  $10^2$  c.f.u.  $\text{ml}^{-1}$  respectively, with 1 h void restriction (VR) to ensure equal basis for establishing infection. At 7 days post-infection (dpi), 94% of pigs (17 of 18) had developed bacteriuria with an average level of bacteriuria  $>10^3$  c.f.u.  $\text{ml}^{-1}$  for all groups (Fig. 1). Only in one pig, from the group that had been infected with the highest inoculum ( $10^8$  c.f.u.  $\text{ml}^{-1}$ ), was bacteriuria not detectable at 7 dpi. At 14 dpi 16 of 18 pigs had bacteriuria above  $>10^3$ . One animal from the group infected with  $10^8$  and  $10^2$  respectively, had only minor bacteriuria ( $10^1$  c.f.u.  $\text{ml}^{-1}$ ) at 14 dpi. There was no significant difference in the average level of bacteriuria between groups at 7 dpi or 14 dpi.

Additionally, four pigs were inoculated with  $10^2$  c.f.u.  $\text{ml}^{-1}$  without VR to evaluate whether successful infection was dependent on VR. At 7 dpi all these pigs had developed bacteriuria at levels indistinguishable from the other groups. In three pigs, the bacteriuria persisted until termination at day 14 (mean  $1.1 \cdot 10^5$  c.f.u.  $\text{ml}^{-1}$ ). At this time point, one pig had cleared the infection indicated by a sterile urine and the average level of bacteriuria was significantly lower compared to pigs infected with VR ( $10^2$ ) (Fig. 1).

The urine concentration of infected pigs, measured by urine specific gravity (density, hereafter USG), was significantly reduced at 7 dpi (mean 1.010,  $P<0.0001$ ) and 14 dpi (mean 1.011,  $P=0.0025$ ) compared to baseline (mean 1.018) (Fig. 1c).

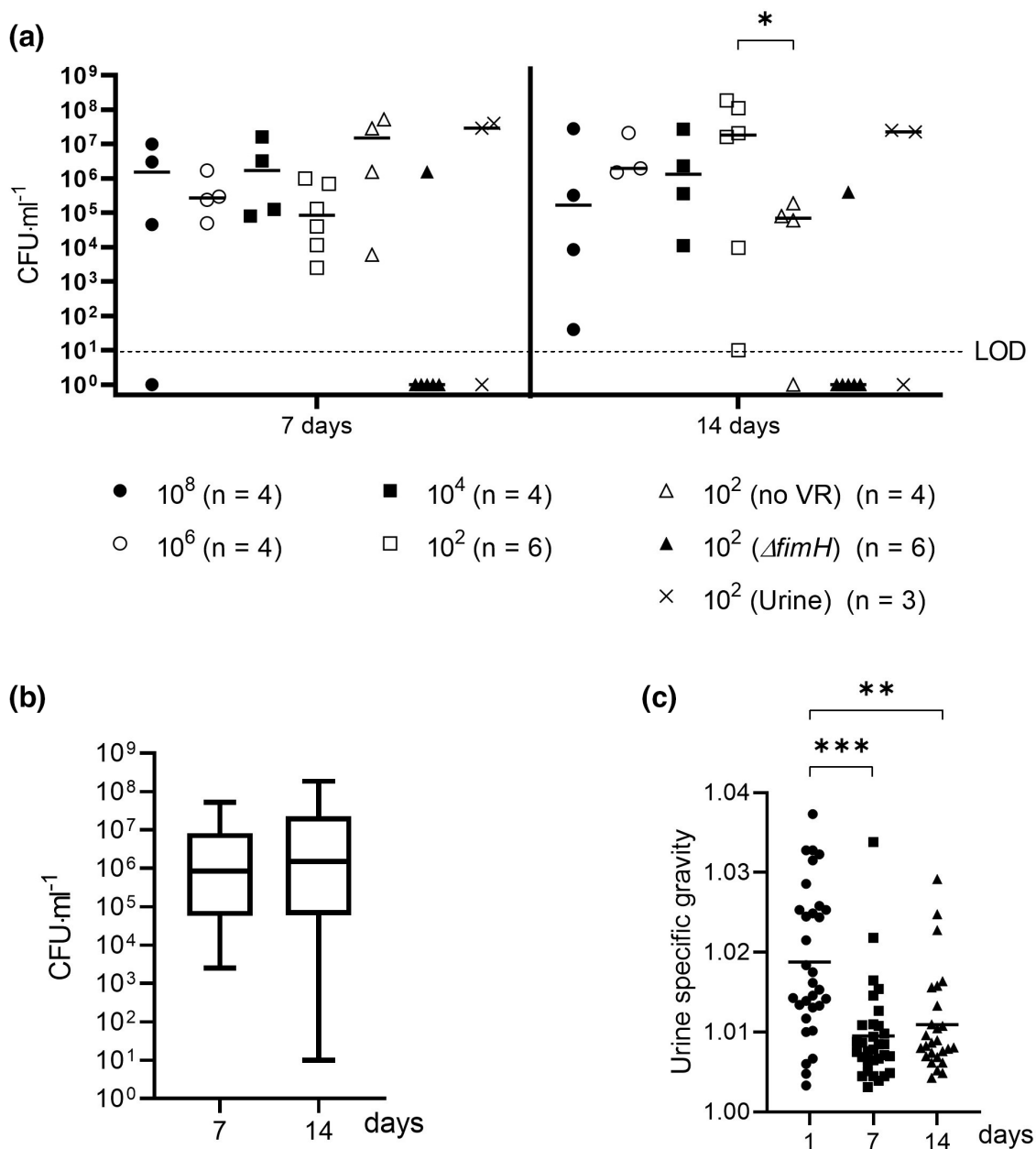
### Initial colonization of the porcine bladder is strongly dependent on T1F

To assess the importance of T1F for cystitis in pigs when exposed to low UPEC inoculation, we infected six pigs with the T1F deficient mutant, UTI89 $\Delta$ *fimH*, in concentrations of  $10^2$  c.f.u.  $\text{ml}^{-1}$  for comparison with the wild-type strain. UTI89 $\Delta$ *fimH* only colonized the porcine bladders in 16.6% (1 of 6) of cases (Table 1). This probability of infection was significantly smaller ( $P=0.015$ ) compared to the wild-type strain which successfully infected 100% (6 of 6) of pigs (Table 1). The pig that did become infected with UTI89 $\Delta$ *fimH* developed bacteriuria of more than  $10^5$  c.f.u.  $\text{ml}^{-1}$  at 7 and 14 dpi comparable to the pigs infected with the wild-type strain. The bacteria in the urine of this pig were confirmed to be *E. coli* devoid of *fimH* by PCR (data not shown).

UTI89 grown statically in pooled porcine urine (four donors) expressed little to no T1F (Table 1) congruent with previous results from growth in pooled human urine [4]. We infected three pigs with  $10^2$  c.f.u.  $\text{ml}^{-1}$  UTI89 preincubated in porcine urine to assess if situational low expression of T1F would attenuate the infectious potential of the wild-type strain, which is otherwise fully capable of expressing functional T1F. UPEC pregrown in porcine urine tended to be less infectious as only 66% (2 of 3) pigs became successfully infected compared to 100% (6 of 6) for UTI89 that was preincubated statically in LB broth to ensure optimal T1F expression, however, this difference was not statistically significant ( $P=0.3$ ).

### Pigs produce anti-UTI89 antibodies in response to infection of the bladder

Blood samples from infected pigs were analysed for specific anti-UTI89 IgG using whole-cell ELISA assays to quantify the level of humoral inflammatory response to infection. For comparison, pigs that had developed bacteriuria at either 7 or 14 dpi (or both) were grouped as 'Infected' ( $n=25$ ), whereas pigs that had not developed bacteriuria, mainly pigs infected with UTI89 $\Delta$ *fimH*, were grouped as 'Non-infected' ( $n=6$ ). For the Infected pigs, the mean anti-UTI89 IgG titre-ratio, compared to day 0, was significantly increased to 2.4 at 7 dpi ( $P=0.0056$ ) and 14.9 at 14 dpi ( $P=0.0008$ ) indicating that infected pigs responded by raising antibodies (Fig. 2). The only exception was the single pig that had developed



**Fig. 1.** Bacteriuria and urine specific gravity over the course of infection. Thirty-one pigs were experimentally infected with UT189 at different inoculum titres (from 10<sup>8</sup> to 10<sup>2</sup> c.f.u. ml<sup>-1</sup>) and monitored for bacteriuria at 7 and 14 days post-infection (dpi). Inoculum was prepared from cultures of Lysogeny Broth or pooled porcine urine (Urine). (a) Pigs were highly susceptible to UTI even at low inoculum titres (10<sup>2</sup>) and the inoculum titre did not significantly influence mean c.f.u. counts in collected urine samples. At 14 dpi, the average bacteriuria level of pigs infected without void restriction (VR) was significantly lower compared to animals infected with VR (10<sup>2</sup>),  $P=0.0495$ . (b) Pooled c.f.u. data showed a high general c.f.u. level in infected pigs, independent of initial inoculum titre, with 25th percentile of 5.7·10<sup>4</sup> c.f.u. ml<sup>-1</sup> and 6.0·10<sup>4</sup> c.f.u. ml<sup>-1</sup> for 7 and 14 days, respectively, and a 75th percentile of 8.3·10<sup>6</sup> c.f.u. ml<sup>-1</sup> and 2.2·10<sup>7</sup> c.f.u. ml<sup>-1</sup> for 7 and 14 days, respectively. Except for two outliers at the lower whisker (14 days), all data were above 10<sup>3</sup> c.f.u. ml<sup>-1</sup>. (c) Pooled data of urine specific gravity ranged between 1.003 to 1.037 and was significantly reduced from baseline (day 1) in response to infections. \* $P=0.0495$ , \*\* $P=0.0006$ , \*\*\* $P<0.0001$ . (a) Two-way ANOVA with Sidak's multiple comparisons test, horizontal lines represent median. (c) One-way ANOVA with Tukey's multiple comparisons test. Horizontal lines represent means. c.f.u., colony forming units; VR, void restriction; ns, non-significant; LOD, limit of detection was 10 c.f.u. ml<sup>-1</sup>.



**Table 1.** Type-1 fimbrial status and infectious outcome

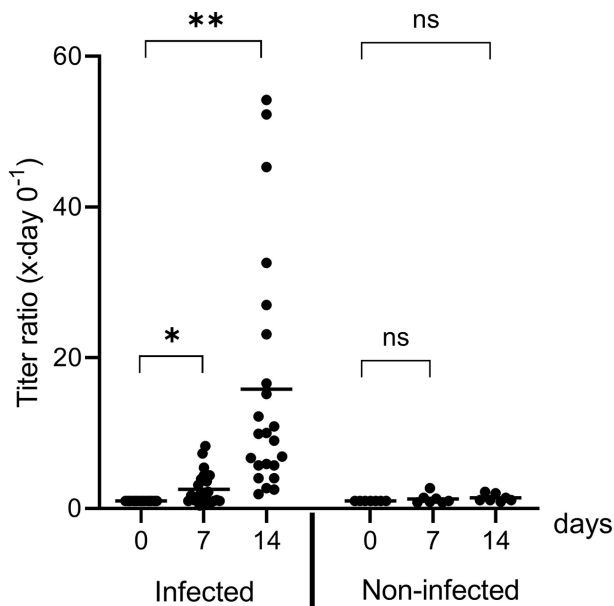
Strain (growth media)	Successful infection*, n (%)	P value	Yeast cell agglutination
UTI89 (LB)	6 of 6 (100)	–	+
UTI89 $\Delta$ <i>fimH</i> (LB)	1 of 6 (16.6)	0.015	–
UTI89 (urine)	2 of 3 (66.6)	0.330	–
UTI89 (from urine sample, 14 dpi)	–	–	–

\*Successful infection:  $>10^3$  colony forming units  $\text{ml}^{-1}$  at seven or 14 days post-infection.  
LB, Lysogeny broth.

bacteriuria, despite being inoculated with UTI89 $\Delta$ *fimH*, as this individual had titre-ratios of 1.0 and 1.1 at 7 and 14 dpi, respectively. Pigs that were Non-infected, i.e. no bacteriuria at 7 or 14 dpi, did not raise antibodies against UTI89, indicated by mean titre-ratio of 1.2 ( $P=0.81$ ) and 1.3 ( $P=0.48$ ) at 7 and 14 dpi, respectively.

### Intracellular *Escherichia coli* survives gentamicin treatment *ex vivo*

We sought to investigate if UPEC were capable of intracellular colonization during infection of the porcine bladder. To do so, the infected bladders were aseptically removed from infected pigs within 10 min post-mortem and immediately washed in sterile saline and cut into smaller samples that were



**Fig. 2.** Humoral response of infected pigs. Pigs were grouped as Infected ( $n=25$ ) or Non-infected ( $n=6$ ) based on the presence of bacteriuria (+/-, respectively). Plasma was analysed for anti-UTI89 IgG antibodies using whole-cell ELISA. Data points represents titre ratio compared to day 0. \* $P<0.01$ , \*\* $P<0.001$ . Repeated measure ANOVA with Tukey's multiple comparisons test. ns, not significant ( $P>0.05$ ).

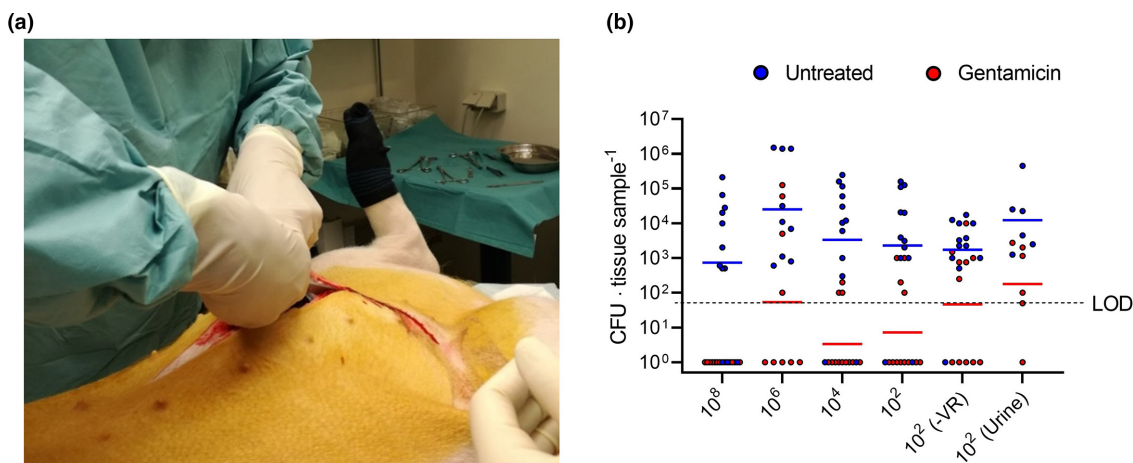
incubated in 6-well microtitreplates with gentamicin or cell culture medium as control. Following incubation, the bladder tissue samples were homogenized and plated to quantify gentamicin-surviving intracellular bacteria [29]. The results are summarized in Fig. 3.

Intracellular bacteria were detected in most groups including the groups of pigs that were not void restricted and the group that were infected with UTI89 preincubated in porcine urine. The only exception was the pigs that were infected with  $10^8$  c.f.u.  $\text{ml}^{-1}$  from which no viable UPEC were detectable in any of the gentamicin-treated samples. Generally intracellular bacteria were only detected in very low numbers, and across all groups approximately 65% of gentamicin-treated samples were sterile. The pigs that were infected with UTI89 pregrown in porcine urine with consequently downregulated T1F showed similar levels of extracellular and intracellular colonization compared to pigs that were infected with UTI89 pregrown in standard LB for optimum T1F expression.

## DISCUSSION

This work represents to our knowledge, the first pathogenesis study of cystitis in a porcine model that shares physiology with humans, as well as general response to infection. Our results provide insight into aspects associated with the initial onset of the infection, which so far has not been obtainable in rodent models or from clinical studies. We found that the infection outcome when introducing UPEC into the pigs' bladder leads to an all-or-nothing situation, i.e. rapid clearance or full colonization. This observation also indicated that a threshold of infection – or minimal infectious dose – exists. UTI is believed to develop in humans also from low inoculum doses, given that humans are highly susceptible and the rate of recurrency is significant. In the current study, we found that as low as  $1 \cdot 10^2$  c.f.u.  $\text{ml}^{-1}$  or a total of  $10^4$  bacteria was adequate to trigger an infection in all animals. This occurred even without void restriction where the vast majority of bacteria may be eliminated soon after inoculation by voiding, leaving only very few bacteria in the bladder that are however still able to mount a robust infection. This puts UPEC in the category of bacteria with the lowest known infectious doses.

Since the porcine model exhibits a high susceptibility to UPEC infection, it allowed us to assess the role of T1F for UPEC to overcome the initial barriers of infection in the urinary tract. Our results demonstrate that T1F expression is highly advantageous for the acute colonization of the urinary bladder in pigs as only one of six pigs became infected upon inoculation with a T1F deficient UPEC compared to six of six inoculated with the wild-type strain. Although these results parallel observations in a study using non-human primates, T1F has not previously been investigated in a low-inoculum model [23]. The one pig that was successfully colonized with UTI89 $\Delta$ *fimH*, showed persistent bacteriuria for 14 days, suggesting that once the initial colonization has been established, T1F are no longer needed for persistence in the urinary bladder. This was further supported with urine samples from



**Fig. 3.** Pigs infected with different inoculum dose from  $10^8$  c.f.u.  $\text{ml}^{-1}$  to  $10^2$  c.f.u.  $\text{ml}^{-1}$  were analysed for tissue-associated bacteria. (a) After 14 days of infection with UTI89, whole bladders from infected pigs were aseptically removed within 10 min post-mortem and punched into 10 mm round-shaped samples (surface area of  $0.8 \text{ cm}^2$ ). (b) From every bladder, three samples were subjected to gentamicin protection assays, while three samples were left untreated. For graphical presentation, data points below the limit of detection were given the value 1 ( $10^0$ ). LOD, limit of detection was 50 c.f.u. per tissue sample. Horizontal lines represent the geometric mean of untreated samples (blue) and gentamicin-treated samples (red). No significant differences were detected between groups using ANOVA with Tukey's multiple comparisons test. c.f.u., colony forming units; LOD, limit of detection; -VR, no voidrestriction; Urine, inoculum was preincubated in urine as opposed to Lysogeny Broth in the other groups.

pigs after 14 days of infection with the wild-type UTI89 as these bacteria expressed minimal T1F levels, consistent with data from the urine of human UTI patients [15]. Our observations that T1F deletion attenuates without completely abolishing UPEC infectious capability, is in accordance with previous studies that demonstrate both adhesive and invasive properties of UPEC strains mutated in the *fim* gene cluster [4, 25, 30].

Infected pigs responded by raising anti-UTI89 antibodies, thus indicating a symptomatic condition. Likewise, pigs inoculated with UTI89 $\Delta$ *fimH* did not produce anti-UTI89 IgG antibodies, thus supporting the absence of disease in these animals. While this might be expected in the pigs that immediately cleared the infection, it was surprising to see that the one pig, which became colonized and reached high bacterial titres of UTI89 $\Delta$ *fimH*, also did not raise an IgG response against the bacterium. This result is based on a single pig and hence conclusions should be made with caution, however the occurrence of this outcome indicates that in the absence of T1F exposed on the bacterial surface, colonization of the bladder can occur without triggering an immune response against any surface epitope on the bacterium (asymptomatic bacteriuria). This result parallels earlier observations in which another UPEC adhesin, PapG of P-pili, was shown to be essential to stimulate protective antibodies *in vivo* [31]. The significance of T1F for generating an antibody response is also in line with the idea that asymptomatic bacteriuria strains have lost their initial ability to produce functional type-1 and P fimbriae in order to persist in the environment of the human bladder without triggering a host immune response [32]. This hypothesis, initially raised by Klemm and coworkers, is based on interesting experimental studies in human patients with asymptomatic *E. coli* strains reconstituted in the *fim* gene cluster

of T1P [32, 33]. However, no experimental infection in humans with pathogenic UPEC strains has been performed as this is unethical and thus unfeasible. This experimental porcine model can provide an intermediate link between humans and rodent/cell culture models that has not previously been available. We conclude, that similar to P-pili, T1F is a key mediator of inflammation and adds to the support, that adhesin-receptor interactions is a primary signalling route for the immune response and may function as an adjuvant to stimulate an immune response against other antigens.

Interestingly, antibody titres, bacterial persistence in the bladder, or late stage c.f.u. levels at 7 and 14 dpi were not influenced by initial inoculum dosage, suggesting that a minimal bladder inoculation leads to complete manifestation of lower UTI in pigs. This also suggests that early stages of colonization represent a key bottleneck in UTI.

It is important to notice that the pigs' urine concentration was within the normal range of human urine (USG=1.003 to 1.030), in contrast to mice that produce highly concentrated urine outside the standard range of humans and pigs (USG=1.045 to 1.064) [34, 35]. Urine concentration is shown to directly influence UPEC growth morphology and biofilm formation suggesting that this parameter is likely to influence the course of UTI [25, 36, 37]. This is further supported by new research demonstrating that the high interstitial sodium concentration in the renal medulla is important for generating an environment with enhanced antibacterial immunity against UPEC, and patients with urine concentrating defects are reported to be susceptible to ascending UTI [38]. Thus, the dilute urine of pigs and humans may, in part, explain their natural susceptibility to UTI. Surprisingly, we discovered that the urine concentration

was significantly reduced at 7 and 14 dpi suggesting that the pigs naturally respond to UTI by increasing their urine production. While the reason for this needs to be investigated more to draw any conclusions, it is intriguing to speculate that UTI may elicit a physiological response that promotes water intake and consequently diuresis – the main doctors advice given to UTI patients - perhaps as conserved physiological mechanism elicited to help clear the infection.

A central paradigm proposed to explain recurrent UTIs by UPEC, involves uroepithelial cell invasion and formation of intracellular bacterial colonies [14]. Although extensively studied *in vitro* and in murine models, the extent of intracellular invasion and whether or not this pathogenic mechanism is involved in human UTI is debated and struggles to win complete acceptance in the clinical societies [39, 40]. The detection of viable bacteria following gentamicin treatment *ex vivo* in this study, indicates that UTI89 may translocate to intracellular niches during UTI in pigs. However, given that most tissue samples were sterile we conclude that intracellular UTI89 are only sparsely distributed in the urothelium at 14 dpi. The observation that none of the pigs inoculated with the highest bacterial titre ( $10^8$  c.f.u. ml<sup>-1</sup>) harboured any detectable invasive bacteria could indicate that a massive inflammatory reaction and exfoliation of superficial BECs had occurred. Since no microscopical analyses were performed to verify the intracellular location, we cannot rule out the possibility that surviving bacteria may be mucous-embedded which might protect them from the gentamicin. Whether these surviving bacteria contribute to persistent bacteriuria or recurrent UTI remains unclear.

While offering new possibilities for studying UTI pathogenesis the model also has limitations. Since pigs are not used widely in animal experiments, fewer reagents to analyse e.g. physiological responses are available for this animal and few genetically modified pig strains exists compared to rodent models. Moreover, the animal is much more costly to use and requires special facilities and trained personnel to handle. This is limiting to the sample size feasible to include in a study which can weaken the ability to conclude on the results. These factors need to be counted in when using this model over e.g. rodents.

In summary, using an optimized porcine model of cystitis, we provide new insight into the infectious potential of UPEC by showing that very low infectious doses are sufficient for successful infection in pigs. We conclude that this infectious potential is dependent on T1F for overcoming initial bottlenecks of infection and that T1F-host cell interactions is a primary inducer of the immune response.

### Data availability

The data supporting the results of this study will be made available upon request.

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### Conflicts of interest

The authors declare that there are no conflicts of interest.

### Ethical statement

Experiments were approved by the Danish Animal Experiments Inspectorate, license number: 2019-15-0201-01626.

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