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Cocktail of Genetically Diverse Lytic Phages Reduces Uropathogenic *Escherichia coli* Colonization in Mouse Urinary Tract

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ABSTRACT

Urinary tract infections (UTIs) caused by uropathogenic *Escherichia coli* (UPEC) are one of the most common bacterial infections in humans. The rise of multidrug-resistant UPEC strains increases the urgent need for alternative treatment. Two diverse lytic bacteriophages (phages), SR02 and SR04, recently exhibited an *in vitro* anti-UPEC activity. In this study, we reported the interplay among UPEC, phages, and the microenvironment of mammalian urinary tract in UTI phage therapy using both *in vitro* (human bladder cell line) and *in vivo* (murine acute UTI) models. A gentamicin protection invasion assay was performed in UPEC-infected human bladder cells (UM-UC-3). Both monophages and the phage cocktail significantly reduced UPEC invasion into UM-UC-3 with a synergistic effect between SR02 and SR04. Female C57BL/6 mice were transurethrally infected with 10^7 colony-forming units of UPEC, and 2 hours later, 10^8 plaque-forming units of monophages and cocktail were single transurethrally administered to the mouse bladder. At 24 h post-UPEC infection, the cocktail significantly reduced UPEC colonization in the mouse bladder and kidney, but not in the urine. The synergism between SR02 and SR04 was observed only in the mouse bladder. Both monophages and cocktail markedly reduced UPEC ascension into mouse kidneys without a synergism or robust tissue proinflammatory cytokine gene expression. However, increased polymorphonuclear cell infiltration was observed in the bladders of SR04-treated mice. In conclusion, we report the contribution of different host urinary tract microenvironments (urine, bladder, and kidney) in the outcomes of UTI phage therapy with two lytic phages and their combination.

Keywords: Uropathogenic *Escherichia coli* (UPEC), urinary tract infection (UTI), bacteriophage (phage) therapy, multidrug-resistant (MDR) UPEC, phage cocktail, mouse model for acute UTI, urinary tract microenvironment

INTRODUCTION

Uropathogenic *Escherichia coli* (UPEC) is a Gram-negative, rod-shaped bacterium in the family Enterobacteriaceae that plays a significant role in bacterial urinary tract infections (UTIs). UTI is one of the most common bacterial infections in humans. Globally, it has been estimated that about 400 million cases with more than 200,000 deaths from UTIs [1]. More than 50% of adult women experience at least one episode of UTIs in their lifetime [2]. This discrepancy is due to anatomical (relatively short urethra) and physiological differences (sex hormones and genitourinary mucosal immunity) between males and females [3]. However, there has been no sex-based difference in age-standardized mortality rate for men or women over 60 years, especially in high socioeconomic level societies [3]. This indicates the significance of UTIs in both sexes, especially in an aging human society.

In a healthy person, UPEC can live harmlessly as a part of the commensal microbiota in the distal gut. Nonetheless, the translocation of UPEC from the intestinal to the urogenital microenvironment (cross-contamination) is believed to be a significant mode of UPEC transmission and a source of recurrent UTI (rUTI) [4]. rUTI is defined as three or more episodes of UTIs within one year and typically caused by the same bacterial strains, indicating the host serves as a persistent reservoir for infection. UPEC is a facultative intracellular bacterium that can replicate and form specialized biofilm-like structures within the bladder urothelium, namely intracellular bacterial communities (IBCs), which protect UPEC from antibiotics and host defenses. The persistence of UPEC in the bladder reseeds rUTI in susceptible hosts [5].

UTIs can be classified as “uncomplicated” and “complicated” UTIs. Uncomplicated UTIs generally occur in healthy, non-pregnant individuals with no structural or functional urinary tract abnormalities, are confined to the lower urinary tract (urethra and bladder), and may occasionally present as asymptomatic bacteriuria (ASB). Acute cystitis, the inflammation of the bladder, is the

most common form of the lower genitourinary tract infection. In contrast, complicated UTIs are characterized by an individual's structural or functional abnormalities of the urinary tract (such as obstruction, stones, or indwelling catheters), and underlying risk factors also increase the risk of infection or reduce the efficacy of antibiotic treatment. Complicated UTIs encompass a broader clinical spectrum ranging from pyelonephritis (inflammation of the kidney) to urosepsis and are often associated with multidrug-resistant (MDR) organisms, treatment failure, and rUTIs [6]. The treatment of complicated UTIs caused by the emergence of MDR UPEC strains, especially extended-spectrum beta-lactamase (ESBL)-producing UPEC, is now challenging [7] [8] [9] [10], necessitating the urgent need for novel therapeutic alternatives [11].

Among the alternatives for the prevention and treatment of UTIs in the post-antibiotic era, bacteriophage (or phage) therapy is among the most promising strategies [12]. Phages are the most abundant particles found in environments where their bacterial hosts live. Phages can be categorized as lysogenic (temperate) or lytic (virulent) phages depending on their life cycle [13]. The specificity of lytic phages in targeting their bacterial hosts reduces colonization of bacterial pathogens without disturbing beneficial microbiota, thereby preventing collateral damage of broad-spectrum antibiotics to the host's health. This advantage of lytic phage has long been proposed as a potential intervention for the prevention and treatment of MDR bacterial infections in animals and humans [14]. Previous studies have already demonstrated the efficacy of using UPEC lytic phages in the treatment of UTIs, as recently reviewed [15]. For instance, an *in vitro* study on UPEC phages in the families *Myoviridae* and *Siphoviridae,* isolated from wastewater, demonstrated that phages can reduce colony counts, biofilm formation, and adherence to human epithelium in several UPEC strains [16]. However, the interactions among the phage, the bacterial host, and the mammalian urinary tract microenvironments in UTI phage therapy remain poorly understood. Our previous study reported the *in vitro* anti-UPEC activity of two genetically diverse UPEC phages, SR02 and SR04, isolated from freshwater canals in Thailand. Both phages are purely lytic and genetically distinct,

belonging to the *Kuravirus* and *Kayfunavirus* families, respectively [17]. In this study, we reported the contribution of the mammalian (murine) urinary tract microenvironments (urine, bladder, and kidney) on the anti-UPEC activity of monophages (SR02 and SR04) and the combination cocktail. The synergistic interaction between SR02 and SR04 was also reported. Both the *in vitro* human bladder epithelial cell line and the *in vivo* murine model of acute UTI were used to investigate the role of the host innate immune response to UTI phage therapy.

RESULTS

Treatment with phage SR02, SR04, and the combination cocktail reduces UPEC invasion to human bladder urothelium

The strains of UPEC and phages used in this study are shown in Supplementary Table 1. A gentamicin protection invasion assay was used to determine the therapeutic efficacy of phage SR02, SR04, and the combination cocktail (1:1 ratio of SR02 and SR04) in UPEC-infected human bladder urothelium (UM-UC-3). The schematic study of the cell infection experiment is depicted in the Supplementary Figure 1. Our data revealed that all phage-treated UM-UC-3 cells had significantly lower recovered invading population (intracellular) UPEC burdens (colony-forming unit (CFU)/mL) than the SM buffer (untreated) control group. (Figure 1a). Monophage SR04 or the combination cocktail significantly reduced UPEC invasion into UM-UC-3 compared to monophage SR02. The synergistic anti-UPEC effect between SR02 and SR04 was also observed. These data indicate that SR02 and SR04 differ in their anti-UPEC activity when interacting with human bladder urothelial cells *in vitro*.

Then, quantitative polymerase chain reaction (qPCR) was used to detect fold changes in proinflammatory cytokine gene expression in UPEC-infected and uninfected UM-UC-3, with or

without phage treatment. The primer lists are shown in Supplementary Table 2. The fold changes of the significant human proinflammatory cytokine genes, including interleukin (*IL)-8*, macrophage inflammatory protein (*MIP)-3*, *IL-1β*, *IL-6*, and tumor necrosis factor (*TNF)-α,* were determined (Figure 1b, c, d, e, and f, respectively). Our data reveal the variations in gene expression among the phage-treated groups. Monophage SR04 and the cocktail significantly upregulated *MIP-3* and *IL-1β* in UPEC-infected UM-UC-3. Only the cocktail-treated group showed a slight increase in *IL-6* expression in UPEC-infected UM-UC-3 compared to the untreated group. However, there were no significant differences in *IL-8* and *TNF-α* expressions between all phage-treated and untreated groups. Neither monophage (SR02 or SR04) nor the cocktail combination induced robust expression of proinflammatory cytokine genes in uninfected human bladder urothelium.

Phages SR02 and SR04 reduce UPEC colonization in the mouse bladder and kidney, but not in the urine

Next, we performed the *in vivo* study using a murine model of acute UTI. The schematic illustration for the animal experiment is shown in Figure 2a. We found no significant difference in UPEC burden in the urine of all groups of mice (Figure 2b). However, UPEC-infected mice treated with the phage cocktail showed a remarkable decrease in UPEC numbers in their bladders and kidneys compared to untreated mice (Figure 2c and 2d, respectively). Interestingly, the phage cocktail demonstrated synergism between SR02 and SR04 in reducing UPEC bladder colonization (Figure 2c). All the phage-treated mice demonstrated significantly lower UPEC burdens in their kidneys than the untreated mice (approximately 3 log10) (Figure 2d). These data suggest contributions from the mammalian urinary tract tissue microenvironment and the synergistic interaction between SR02 and SR04 against UPEC bladder colonization. Surprisingly, neither SR02 nor SR04 can significantly reduce planktonic UPEC in mouse urine; however, both can significantly reduce tissue-associated UPEC levels, especially in the kidney. This suggests that UTI phage

therapy might be less effective against planktonic cells than against tissue-associated UPEC in the mammalian urinary tract.

The presence of phage SR02 and SR04 in mouse urine, bladder, and kidney after transurethral administration

The presence of viable phage SR02 and SR04 in mouse urine and urinary tract tissues (bladder and kidney) was determined by a double-layer plaque assay. Our results show that monophage SR04 and the cocktail can be significantly recovered from mouse urine, bladder, and kidney at higher PFU counts than monophage SR02 (Figure 3a, b, and c, respectively). Interestingly, viable SR04 and the cocktail (mixture of SR02 and SR04) can be detected at approximately the same amount in mouse urine, but not in the mouse bladder or kidney. SR02 exhibited the lowest PFU counts in mouse urine and kidney compared to SR04 and the cocktail. To determine whether high phage counts correlated with low UPEC counts in mouse urinary tract, Pearson's correlation was used (Supplementary Figure 2). There was only one negative correlation ($r = 0.968$) between phage PFU and UPEC CFU counts in the kidney of monophage SR04-treated mice (Supplementary Figure 2h), but not in SR02- or cocktail-treated mice. These indicate the contribution of phage strain-specificity to the availability and correlation between phage and UPEC numbers within different mammalian urinary tract microenvironments (urine, bladder, and kidney).

No significant alteration in mouse bladder and kidney proinflammatory cytokine gene expressions after the phage treatment

The innate immune response in mouse urinary tract tissues (bladder and kidney) was determined by fold changes in the expression of significant proinflammatory cytokine genes. The mRNA expression fold changes of keratinocyte chemoattractant (*Kc*), inducible nitric oxide synthase

(*Nos2*), *IL-6*, *Mip2,* and *Tnf-alpha* in the mouse bladders and kidneys were measured by qPCR. UPEC-infected mice exhibit a significant increase in proinflammatory cytokine genes in their bladder and kidney tissues (Figures 4 and 5, respectively) compared to the mock-infected mice (except kidney *Nos2* in Figure 5b). Our data reveal no significant difference in the fold change of these proinflammatory cytokine genes between bladders and kidneys of phage-treated and untreated mice.

Treatment with monophage SR04 increases polymorphonuclear cell (PMN) infiltration in the bladder, but decreases in the kidney of mice

In addition to fold changes in mRNA levels of proinflammatory cytokine genes determined by qPCR, the inflammatory responses in the mouse bladder and kidney were also evaluated microscopically by histopathological analysis. Haematoxylin and eosin (H & E) staining of mouse bladder and kidney tissues was performed, and the slides were scored by a veterinary pathologist in a double-blind manner. The total bladder histopathological scores of all groups of mice were assessed using the criteria shown in Supplementary Table 3. UPEC infection resulted in increased polymorphonuclear (PMN) cell infiltration and total histopathological scores in the mouse bladder and kidney at 24 h post-infection (Figure 6). However, none of the phage treatments significantly reduced PMN infiltration or total scores in the bladders of UPEC-infected mice. On the contrary, there was a statistically significant increase in bladder PMNs in the monophage SR04-treated mice (with a large variation of PMN counts in the bladder of the SR02-treated mice), but not in the cocktail-treated mice (Figure 6a). Nonetheless, SR04-treated mice showed the lowest kidney PMN counts, whereas none of the phage-treated mice significantly reduced kidney PMN infiltration or total scores (Figure 6c and 6d, respectively). Representative histopathological images of the mouse bladders and kidneys from each group are shown in Figures 7 and 8, respectively.

DISCUSSION

UPEC is the most common cause of bacterial UTI and extra-intestinal *E. coli* infection in humans. There are two major clinical manifestations of acute UPEC UTIs in humans: acute cystitis and pyelonephritis, in which patients develop inflammation of the bladder and kidney, respectively. Although most patients infected with UPEC have self-limited episodes, high-risk groups of patients, especially those with an immunocompromised status, abnormal structure of the urinary tract, extreme ages, or who are catheterized, might develop serious complications (*e.g.,* renal scarring, renal failure, or urosepsis) [18] [19]. According to the WHO's bacterial priority pathogens list in 2024, carbapenem- and third-generation cephalosporin-resistant Enterobacterales, including UPEC, have been classified as a critical group of human pathogens that require prioritized research and development of interventions [20]. As we approach a global wave of aging human societies and the post-antibiotic era, alternative interventions for this common bacterial infection are essential [21]. Among these approaches, phage therapy is a promising strategy for treating human UTIs caused by MDR and might be a sole treatment option for extensively drug-resistant (XDR) UPEC strains [22] [23] [12].

The interaction among phage, UPEC, and the host immune system in the urinary tract plays a significant role in determining the efficacy of UTI phage therapy. To overcome the problematic development of phage-resistant bacteria after phage treatment, the use of a combined or phage cocktail has often been discussed [24]. The influence of phage-phage interaction between different phage genera, phage binding sites (receptors), and formulations plays a critical role in phage therapy efficacy [25]. Previously, we reported the *in vitro* anti-UPEC activities of two novel, genetically distinct lytic UPEC phages isolated from freshwater canals in Thailand. Those two lytic phages belong to different genera of *Kuravirus* and *Kayfunavirus*, named SR02 and SR04, respectively [17]. In this study, we report the anti-UPEC and immunomodulatory activities of phages

SR02 and SR04, as well as their combination cocktail, when encountered with the mammalian urinary tract microenvironment using both cell culture and a transurethral mouse model of acute UTIs.

One crucial step of UPEC pathogenesis in its mammalian urinary tract is bladder cell invasion. UPEC virulence factors, such as alpha-hemolysin and cytotoxic necrotizing factor-1, enable UPEC to invade and survive within the host bladder urothelium [26]. Intracellular UPECs can replicate and escape from otherwise robust host defenses, especially neutrophils [27]. In this study, we determined the efficacy of SR02, SR04, and a cocktail (1:1 mixture of SR02 and SR04) in reducing UPEC invasion of human bladder urothelium (UM-UC-3) using a gentamicin protection invasion assay. We found that giving either phage or the cocktail after UPEC infection significantly reduces UPEC invasion into human bladder urothelium. A difference in efficacy between the tested phages in attenuating UPEC invasion is observed. SR04 (*Kayfunavirus*) and the combination cocktail reduced UPEC invasion into human bladder urothelium better than SR02 (*Kuravirus)* alone. This indicates synergistic and strain-specific effects among different phages in reduction of intracellular burdens of UPEC in human bladder urothelial cells. Consistent with our previous report, SR04 is more effective at targeting the UPEC strain UTI89 than SR02 [17]. SR04 showed a larger burst size and faster killing kinetics than SR02. Moreover, the prophylactic administration of SR02 and SR04 reduced UPEC invasion into human bladder epithelium without altering bladder proinflammatory cytokine gene expression.

Here, we found that phages SR02 and SR04 alone, without UPEC infection, did not induce a significant change in the inflammatory response in human bladder urothelium. Instillation of phages SR02 and SR04 alone into human bladder urothelium did not cause significant upregulation of proinflammatory genes (*IL-8*, *MIP-3*, *IL-1β*, *IL-6*, and *TNF-α*). However, treatment of UPEC-infected UM-UC-3 with SR04 alone and the cocktail increased the upregulation of *MIP-3* and *IL-1β*, important

chemoattractant and pyrogenic cytokines in humans, respectively. These data align with some previous reports on the efficacy of phage therapy in inhibiting UPEC-mediated invasion of bladder urothelial cells. For instance, rapidly multiplying UPEC phage of the family *Autographiviridae* vB_EcoP reduces both planktonic and human bladder cell-adhered UPEC at 6 hours post-infection [28]. The unclassified *Caudoviricetes* vB_Eco_ZCEC08 UPEC phage was recently shown to inhibit UPEC invasion, exhibit good physicochemical stability, and have low cytotoxicity to host bladder cells and skin fibroblasts [29]. Phage vB_Ecom-phiEc1 (family *Myoviridae*) and phage vB_EcoS-phiEc3 (family *Siphoviridae*) decrease UPEC adhesion to the human epithelium cell line (HEp-2) cells more effectively than phage vB_EcoS-phiEc4 (family *Siphoviridae*) [16]. Our study confirms that phage exerts strain-specific effects on inhibiting UPEC invasion into human bladder urothelial cells.

Phage therapy could provide therapeutic benefits through two possible mechanisms: (i) direct bacterial lytic activity in the host's environment and (ii) indirect host immune modulation that protects the host from subsequent bacterial infections [30]. The tripartite interaction between phages, pathogens, and their mammalian host immune response plays a critical role in the outcomes of phage therapy [12]. Our data show that monophage SR02 or SR04 and the cocktail significantly decreased UPEC numbers in the mouse kidney by approximately 4 log10 compared to the untreated mice. However, only the phage cocktail-treated mice, and not monophage-treated mice, exhibited significantly lower numbers of UPEC (about 5 log10) in their bladders compared to the untreated mice. Phage SR04 and the phage cocktail were detected in mouse urine, bladder, and kidney at higher levels than SR02 alone, suggesting that SR04 is more adept than SR02 in colonizing the mammalian urogenital tract, specifically the bladder and kidney. Lower SR02 phage detection in mouse urine, bladder, and kidney might be due to several factors, such as elimination by the host immune system (immune clearance), reduced specificity between the phage and its bacterial host, or reduced bacterial host numbers [31]. Moreover, a negative correlation between

phage and UPEC colonization was observed only in the kidneys of mice treated with SR04. Our data show a significant reduction in kidney UPEC burden in all phage-treated groups, but not in the bladder (only in the cocktail-treated group). These suggest that the phage-host tissue interaction might contribute to the outcome of phage therapy, especially the microenvironment between the mouse bladder and kidney.

The efficacy of phage therapy against bacterial UTIs in a mouse model has already been reported in several studies [32] [33] [34] [35] [36]. However, there are relatively few studies focusing on UPEC UTI phage therapy. For example, Nishikawa et al. reported that the T4-phage-like KEP10 phage, when administered peritoneally to UPEC-infected mice, resulted in a marked decrease in mortality rate [32]. This study by Nishikawa also indicates the advantage of the intraperitoneal route of phage administration in mice over the more complex transurethral route. The UPEC phage KEP10, when administered peritoneally, significantly reduced mortality in mice infected with UPEC, and KEP10 alone did not cause adverse effects in mice. The combination of genetically distinct phages from the families *Chaseviridae*, *Myoviridae*, and *Podoviridae* exhibited high efficacy in killing UPEC and pathogenic intestinal *E. coli* by recognizing distinct lipopolysaccharide molecules on various *E. coli* hosts [37]. Bhargava et al. reported that transurethral administration of a high-dose (200 µL of 10^7 and 10^8 PFU/mL) UPEC phage cocktail into the bladder of UPEC-infected rats resulted in the successful cure of UTI [38].

To investigate the role of phages SR02 and SR04 on the host innate immunity in the mouse urinary tract, the fold changes in mRNA expressions of the important proinflammatory cytokines and mediators (*Kc*, *Nos2*, *IL-6*, *Mip-2,* and *Tnf-alpha*) were determined by qPCR. Lytic phages SR02 and SR04 did not significantly alter the expression of these genes in either bladder or kidney tissues compared to untreated mice. No significant differences in mRNA fold changes for these proinflammatory genes were observed between the monotherapy and combination cocktail-treated

groups. However, there was an increased expression of *Tnf-alpha* in the UPEC UTI89-infected mouse bladder and kidney, but not in human bladder UM-UC-3 cells**.** This difference might be due to differences in the timing of immune responses between the mouse and cell culture experiments. Interestingly, our data show no direct correlation between increased UPEC numbers in tissues (bladder and kidney) and the upregulation of these proinflammatory genes. In contrast, a previous report on phage therapy against *Cronobacter* UTIs noted that phage therapy can reduce the numbers of *Cronobacter* in the kidney with attenuating the gene expression of *Tnf-α* and *Mcp-1* in mice [36]. The strains of mice also influence the host's immune response to UTI. The UPEC numbers in the bladder and kidneys on day 1 post-infection in C57BL/6 mice could be varied [39] [40]. For example, female C57BL/6J mice rapidly resolve the urine UPEC strain UTI89 numbers within 1-3 days post-infection. In this study, we also found a large variation in urine UPEC UTI89 counts (approximately 10-10^7 CFU/mL) across all UPEC-infected groups. Our data reveal high burdens of UTI89 in the mouse bladder and kidney, at approximately 10^8 and 10^6 CFU/g tissue, respectively. Transurethral treatment with phage SR04 and the combination cocktail significantly reduced UTI89 burdens in the mouse bladder and kidney compared with untreated mice.

In addition to the fold change of tissue proinflammatory genes, we also determined inflammation scores in mouse bladder and kidney tissues as previously described [40]. Surprisingly, histopathological data showed increased PMN infiltration (cells/HPF) and total histopathological scores in the monophage groups (SR02 or SR04) compared with the cocktail-treated and untreated mice. These findings suggest that a single transurethral administration of SR02 or SR04 in UTI89-infected mice leads to increased bladder and kidney inflammation at the microscopic level, with an unknown mechanism. Hence, further investigations are needed to determine the contribution of UPEC phage (single or cocktail) to the inflammation and other immune components of the mouse urinary tract with or without UPEC infection.

CONCLUSION

Lytic phage SR04 (*Kayfunavirus*) is more effective than SR02 (*Kuravirus*), and the phage cocktail exhibits a synergistic anti-UPEC effect both *in vitro* and *in vivo*. Single treatment with SR02, SR04, and the cocktail significantly reduced UPEC invasion into the human bladder cell line. Single transurethral administration of the SR04 and the cocktail significantly reduced the number of UPEC ascending to the mouse kidney without an associated change in proinflammatory gene expression. However, SR04 alone can increase histopathological scores in the mouse bladder but decrease them in the kidney, with an unknown mechanism. This study indicates the potential of SR04 and the combination with SR02 for treating UTI in humans. However, further studies focusing on phage-phage interactions in different cocktail formulations and the consequences of UPEC phage interaction on host bladder and kidney tissues will be essential.

MATERIALS & METHODS

Ethical approvals

The animal experiments of this study were reviewed and approved by the Animal Care and Use Committee, Chiang Mai University, Thailand, in accordance with the Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines (Approval No.2567/MC-0004). This study was also approved by the Institutional Biosafety Committee, Faculty of Medicine, Chiang Mai University (Approval No. CMUIBC02026/2566).

Bacterial strain and culture conditions

Uropathogenic *Escherichia coli* (UPEC) strain UTI89 was originally isolated from an acute cystitis female patient [41]. The strain was grown aerobically (with shaking) at 37°C in Luria-Bertani (LB) broth (Difco, Sparks, MD, US) for 4 h. Then, the bacterial solution was subcultured (1:1,000) in LB broth and statically (without shaking) and grown at 37°C for 16-18 h to promote type-I-pili expression, an important virulence factor of UPEC in the mouse urinary tract [42]. After 16-18 h of incubation, 30 mL of the overnight culture was centrifuged at 4,000 rcf for 10 min at 4°C. Then, the supernatant was discarded, and the bacterial cell pellets were resuspended in sterile phosphate-buffered saline (PBS) to obtain a final concentration of UPEC UTI89 of approximately 2×10^9 CFU/mL. The suspension was then kept on ice until use.

Phage SR02 and SR04 preparation

Two solutions of lytic bacteriophages (SR02 and SR04) specific to UPEC were prepared as in a previous report with slight modifications [17]. Briefly, the full plate double-layer agar (DLA) method (0.35% molten LB top agar poured onto the 1.5% LB bottom agar plate) was performed. Each confluent DLA was added with 5 mL SM buffer (200 mM NaCl, 10 mM $MgSO_4$, and 50 mM Tris-HCl, pH 7.5), and gently swirled for 5 h at room temperature 25°C. Phage lysate was then collected and centrifuged at 4 °C, 4,700 g for 30 mins. The supernatant was filtered through a 0.45 µm polyethersulfone (PES) membrane filter (Whatman, Puradisc 25 mm, Cytiva, Buckinghamshire, United Kingdom). The filtrate was then centrifuged at 4 °C, 20,000 g for 45 mins. The phage pellet was resuspended with 2 mL SM buffer and kept at 4 °C for 16-18 h. Finally, the phage solution was centrifuged at 4 °C, 1,800 g for 15 minutes, and filtered through a 0.22 µm PES membrane filter (Whatman, Puradisc 25 mm, Cytiva, Buckinghamshire, United Kingdom), and stored at 4 °C until use.

Cell culture conditions and human bladder epithelium cell invasion assay

The human bladder epithelium UM-UC-3 (ATCC CRL-1749) was purchased from ATCC and cultivated as previously described [17]. In brief, approximately 5 x 10^5 of UM-UC-3 cells were seeded in each well of a 24-well plate and incubated at 37 °C with 5% CO_2 in a humidified incubator. The complete growth medium was composed of RPMI-1640 supplemented with 2.05 mM L-glutamine (Cytiva, Utah, USA), 10% fetal bovine serum (FBS) (Cytiva, Austria), and 1% penicillin/streptomycin (Cytiva, Austria). To synchronize the cells, the complete media were replaced with RPMI-1640 without FBS and antibiotics-antifungal agents for 24 h. Then, UM-UC-3 cells were infected with 10^7 CFU of UPEC UTI89 (MOI of 20) and incubated at 37 °C for 90 min. Each well was washed twice with 500 µL of Dulbecco's phosphate-buffered saline (DPBS) (Hyclone, Singapore). Then, 10 µL of high-titer phages SR02, SR04, or their combination cocktail (1:1) was added into each well (total 10^7 PFU/well). SM buffer was used as a control vehicle. The plate was gently swirled and incubated at 37 °C with 5% CO_2 for 3 hours, then washed twice with DPBS. Later, 0.5 mL medium containing 100 µg/mL of gentamicin sulfate solution (AppliChem, Germany) was added to each well and incubated at 37 °C with 5% CO_2 for an additional 90 mins to eliminate extracellular UPEC. Finally, cells were lysed with 0.5 mL of 1% Triton-X-1000 (Thermo Fisher Scientific, USA), and the intracellular UPEC UTI89 numbers were counted using a standard serial ten-fold plating method as previously described [43]. Briefly, in the gentamicin protection assay, the extracellular (non-invasive) populations of pathogens were eliminated by gentamicin sulfate, and the host cells were lysed for the determination of intracellular (invasive) populations of the pathogen.

Detection of human bladder urothelial cell gene expressions by a quantitative polymerase chain reaction (qPCR)

Human bladder urothelial cells (UM-UC-3) were seeded into a 6-well plate (density about 10^6 cells per well). Cells were infected with UPEC UTI89 (MOI 20) for 30 mins, and 10^7 PFU phages SR02, SR04, and the combination cocktail were added into each well. The mRNA of UM-UC-3 was collected as previously reported [17]. Briefly, 1 mL of Trizol reagent (Ambion, USA) was added into each well, and RNA extraction was performed following the manufacturer's protocol. The cDNA was generated by using the RevertAid First Strand cDNA reagents (Thermo Fisher Scientific, Lithunia). Then, the qPCR was performed by using the SensiFAST SYBR Lo-Rox Kit (Meridian Bioscience, Tennessee, USA) and ViiA7 Real-Time PCR system (Applied Biosystems). All data was normalized with human *GAPDH* as a housekeeping gene. The fold change of each gene was calculated by the comparative Ct method as previously described [44]. Primers used for the qPCR are listed in Supplementary Table 2.

Animal studies

The animal experiments of this study were reviewed and approved by the Animal Care and Use Committee, Chiang Mai University, Thailand, in accordance with the AAALAC guidelines (Approval No.2567/MC-0004). The study is reported in accordance with the ARRIVE guidelines. Six to eight-week-old female C57BL/6NJcl mice (*Mus musculus*) (weight between 18.00 – 20.0 g) were purchased from Nomura Siam International (Bangkok, Thailand). All mice were acclimatized for 1 week and housed in 12 dark/ 12 light hour cycles in an environmentally controlled room (room temperature between 20-22 °C; humidity between 40 – 60%). Mice were fed with standard mouse chow and drinking water *ad libitum*. Then, mice were divided into four groups (6-7 mice per group): (1) SR02, (2) SR04, (3) cocktail of 1:1 ratio of SR02 and SR04, and (4) SM buffer (untreated control) group. Transurethral administration of UPEC UTI89 and phages was performed as previously described [42]. In short, mice were anesthetized under 2-5% isoflurane during the entire procedure as follows:.A sterile plastic catheter (24G x 3/4", Nipro, Japan) lubricated with the lubricant (KY-jelly,

Dansom Lane, United Kingdom) attached with a 1-mL syringe filled with bacterial solution (total 10^8 cfu/mouse) was gently inserted via the urethral orifice. Each mouse was infected with 50 µL of the UTI89 solution. At two hours post-infection, 50 µL of phage SR02, SR04, and their combination cocktail were transurethrally given to each mouse. SM buffer was used in the untreated control group. At 24 h post-infection, mouse urine was collected, and all mice were euthanized to collect bladder and kidney tissue. The euthanization of mice was performed by CO_2 asphyxiation as a primary method, followed by cervical dislocation as a secondary method.

RNA extraction for animal tissues

At 24 h post-infection, all mice were euthanized, and tissues (bladder and kidney) were collected and kept in RNA preservative solution (RNAstore Reagent; TIANGEN, China). Mouse tissue RNA was extracted by a phenol-chloroform method as described previously [44]. In short, the mouse bladder and kidney were homogenized by 1 mm diameter zirconium/silica beads (BioSpec Products, Oklahoma, USA) in 1mL TRIzol® reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol. Then, 200 µL of chloroform was added to the homogenates and shaken vigorously for 15 sec. The mixture was incubated at room temperature for 10 min and centrifuged at 12,000 g for 15 min at 4 °C. The top aqueous (RNA) phase was then transferred to a new RNase-free 1.5 mL centrifuge tube for RNA precipitation. To precipitate RNA, 500 µL of isopropanol was added to the aqueous phase, and the mixture was incubated at room temperature for 10 min, then centrifuged at 12,000 g for 8 min at 4 °C. The RNA pellet was washed with 1 mL of 75% ethanol before performing cDNA synthesis. The cDNA was synthesized using the RevertAid First Strand cDNA reagent (Thermo Fisher Scientific, Vilnius, Lithuania) for the qPCR experiment.

Determination of UPEC and phage numbers in the mouse urinary tract

Viable counts of UPEC UTI89 in the mouse urinary tract (urine, bladder, and kidney) were enumerated by a serial-dilution plating technique with sterile PBS. Mouse urine was collected before euthanization by gently pushing on the mouse's lower back and collecting the urine on sterile parafilm (at least 30-50 µL per mouse) by pipetting. Mouse bladder and kidney tissues were collected in 1 mL PBS supplemented with 20% glycerol and homogenized in a bead-beating machine using 1 mm diameter zirconium/silica beads (Biospec Products, Bartlesville, US). UTI89 colony counts (cfu/mL urine and cfu/gm tissue) were enumerated with a serial ten-fold dilution technique using LB agar with 0.05 mg/mL kanamycin (AppliChem, Germany), and incubated at 37 °C with the ambient atmospheric gas conditions for 16 h. A double-layer agar assay was used to determine phage SR02 and SR04 titers in mouse urine, bladder, and kidney.

Histopathological study of the mouse bladder and kidney

Segments of the mouse bladder and kidney were fixed in 10% buffered formalin, embedded with paraffin, and stained with hematoxylin and eosin (H&E). The slides were double-blinded scored by a veterinary pathologist. Tissues were examined under a light microscope at 40X magnification using a Nikon advanced upright microscope with a video capture digital camera (ECLIPSE Ni-U, Nikon, Tokyo, Japan). Polymorphonuclear cells (PMNs) in bladder and kidney tissues were counted as previously described [45]. The histological grading scale for the degree of inflammation in mouse bladder and kidney was used with criteria as reported in a previous study (Supplementary Table 3) [40]

Statistical Analysis

Statistical analysis was performed using *GraphPad Prism software V10*. Student's *t*- test was used to analyze the difference between two groups of data, and one-way analysis of variance (ANOVA) with Turkey's test was used for multiple comparisons. *$P < 0.05$ was defined as a statistically significant difference. Bacterial counts and mRNA fold changes were logarithmically transformed before statistical analysis. The Mann-Whitney non-parametric test was used to analyze histopathological scores. The GraphPad Prism version 10.2.0 software was used to generate the graphs. (https://www.graphpad.com)

AUTHOR CONTRIBUTIONS

Conceptualization: PT, PM, VC

Methodology: PT, PM, CS, PN

Validation: PT, PM, VC

Formal analysis: PT, PM, CS, SB

Investigations: PT, PM, CS, WS, SB, IEI

Resources: PT, VC, MHH

Supervision: PT & VC

Visualization: PT, PM, WS

Data Curation: PT, PM, VS

Writing – Original Draft Preparation: PT, PM, WS

Writing – Review & Editing: PT, PM, VC, MHH

Funding acquisition: PT & VC

Project administration: PT

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CONFLICTS OF INTEREST

All the authors declare no conflicts of interest

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and the Supplementary Information files.

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FIGURE LEGENDS

Figure 1. The synergistic effect between SR02 and SR04 against UPEC invasion of the human bladder urothelial cells. UM-UC-3 cells were infected with UPEC UTI89 (MOI of 20) and incubated at 37 °C for 90 min before treatment with phages (total 10^7 PFU). The recovered UTI89 viable cells were counted as CFU/mL (a). The fold changes in pro-inflammatory cytokine gene expression (*IL-8*, *MIP-3*, *Il-1β*, *IL-6* and *TNF-α)* in each group are shown (b, c, d, e, and f, respectively). Bars represent the geometric mean with geometric standard deviation of at least three independent experiments as error bars. *, **, ***, **** indicate *P*-value < 0.05, 0.01, 0.001, and 0.0001, respectively; ns, a non-statistically significant difference.

Figure 2. Transurethral administration of phages SR02, SR04, and their combination cocktail reduced UPEC UTI89 colonization in the mouse bladder and kidney but not in the urine. The schematic illustration of the mouse UTI model is shown. Phages were transurethrally given to UTI89-infected mice at 2 h after infection, and mice were euthanized at 24 h post-infection (a). No significant differences were observed in UTI89 levels in mouse urine (b). A cocktail of phage SR02 and SR04 exhibited the highest efficacy in reducing UTI89 colonization in the mouse bladder compared to the monotherapy groups (c). All phage-treated groups illustrated a significant reduction in UTI89 colonization in the mouse kidney (d). Bars represent the geometric mean with a geometric

standard deviation as error bars. *, **, **** indicate *P*-value < 0.05, 0.01, and 0.0001, respectively, ns, a non-statistically significant difference.

Figure 3. Persistence of phages SR02 and SR04 in mouse urine and urinary tract (bladder and kidney) after transurethral administration. Phage SR04 and the cocktail of SR02 and SR04 were detected at a higher level compared to that of SR02 in mouse urine (a). The level of phage (PFU/gm tissue) SR04 alone was higher than that of SR02 and the combination cocktail in the mouse bladder (b) and kidney (c). Each dot represents an individual mouse. Bars represent the geometric mean with a geometric standard deviation as error bars. **, **** indicate *P*-value < 0.01, and 0.0001, respectively, ns, a non-statistically significant difference.

Figure 4. No significant alteration in mouse bladder proinflammatory cytokine gene expression was observed between phage-treated groups and the untreated group. Fold changes of mRNA expression of proinflammatory genes *Kc*, *Nos2*, *IL-6*, *Mip2,* and *Tnfa* (a, b, c, d, and e, respectively) were determined by a qPCR and normalized to mouse *Gapdh* by a comparative *Ct* method. Each dot represents an individual mouse. Bars represent the geometric mean with a geometric standard deviation as an error bar. ** indicates *P*-value < 0.01, and ns indicates a non-statistically significant difference.

Figure 5. No significant fold change in mouse kidney proinflammatory cytokine gene expression in phage-treated groups compared to the control group. Fold changes of mRNA expression of proinflammatory genes *Kc*, *Nos2*, *IL-6*, *Mip2,* and *Tnfa* (a, b, c, d, and e, respectively) were determined by a qPCR and normalized to mouse *Gapdh* by a comparative *Ct* method. Each dot represents an individual mouse. Bars represent the geometric mean with a geometric standard deviation as an error bar. *, **, *** indicate *P*-value < 0.05, 0.01, and 0.001, ns indicates a non-statistically significant difference.

Figure 6. Haematoxylin & eosin (H & E) staining-based mouse bladder tissue histological studies of polymorphonuclear cell (PMN) infiltration and total histopathological scores. UPEC UTI89 infection resulted in increased mouse bladder PMNs infiltration and total histopathological scores (a and b, respectively) compared to mock infection. The highest levels of bladder PMN infiltration and total histopathological scores were observed with SR04 monotherapy. Phage SR04 decreased PMN infiltration in the mouse kidney (c) but not the total histopathological score (d). Each dot represents an individual mouse. Bars represent the mean with a standard error of the mean (SEM) as an error bar. *, **, *** indicate *P*-value < 0.05, 0.01, and 0.001, ns indicates a non-statistically significant difference. (Mann-Whitney non-parametric test)

Figure 7. Representative histological images of mouse bladder H & E staining for UTI89-infected mice treated with SR02 (a), SR04 (b), cocktail (SR02 + SR04) (c), SM buffer (untreated) (d), and mock infection with PBS (e). All pictures were taken under a light microscope at 40X magnification. The scale bar is 250 μm.

Figure 8. Representative histological images of mouse kidney H & E staining for UTI89-infected mice treated with SR02 (a), SR04 (b), cocktail (SR02 + SR04) (c), SM buffer (untreated) (d), and mock infection with PBS (e). All pictures were taken under a light microscope at 40X magnification. The scale bar is 250 μm.

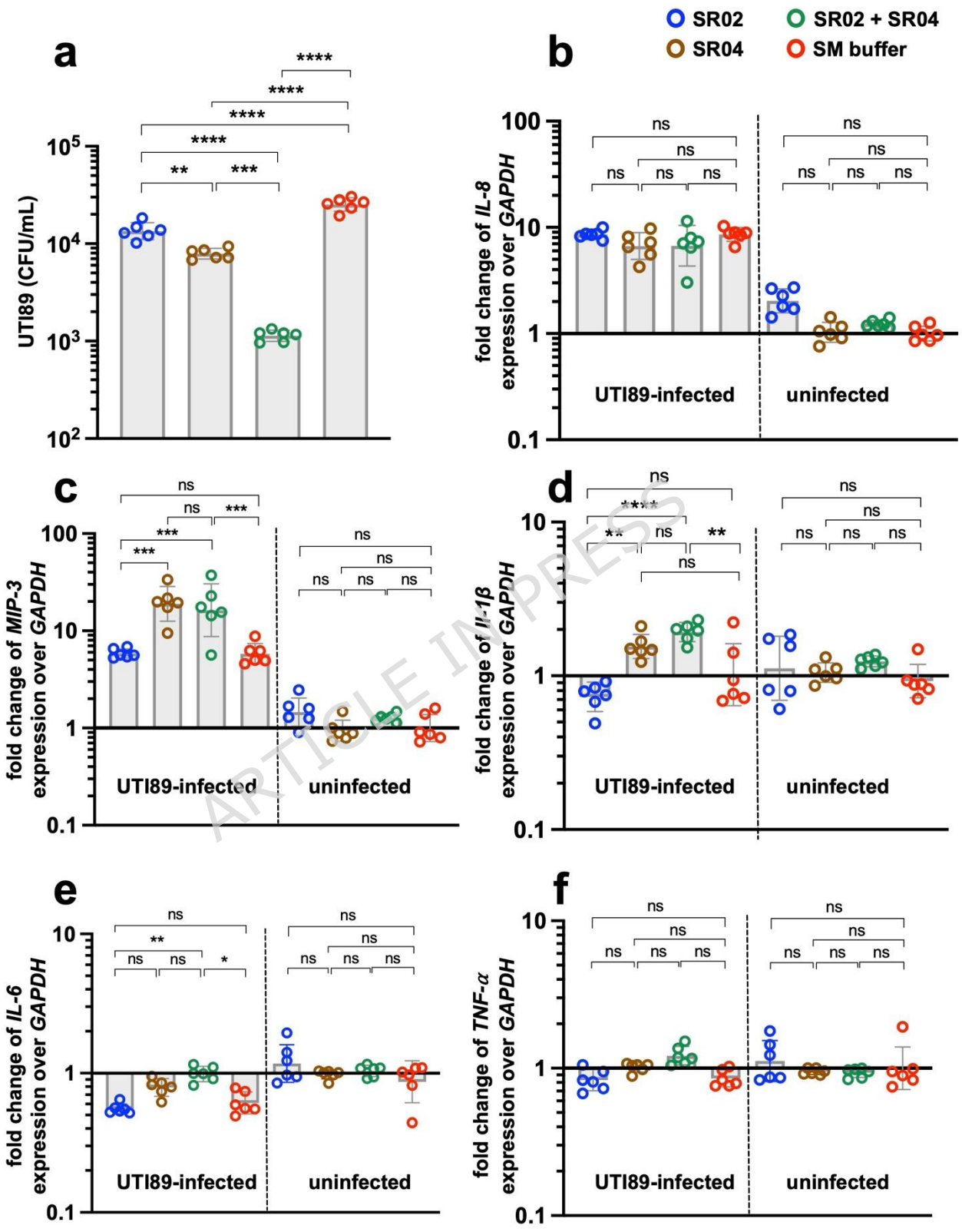

Figure 1

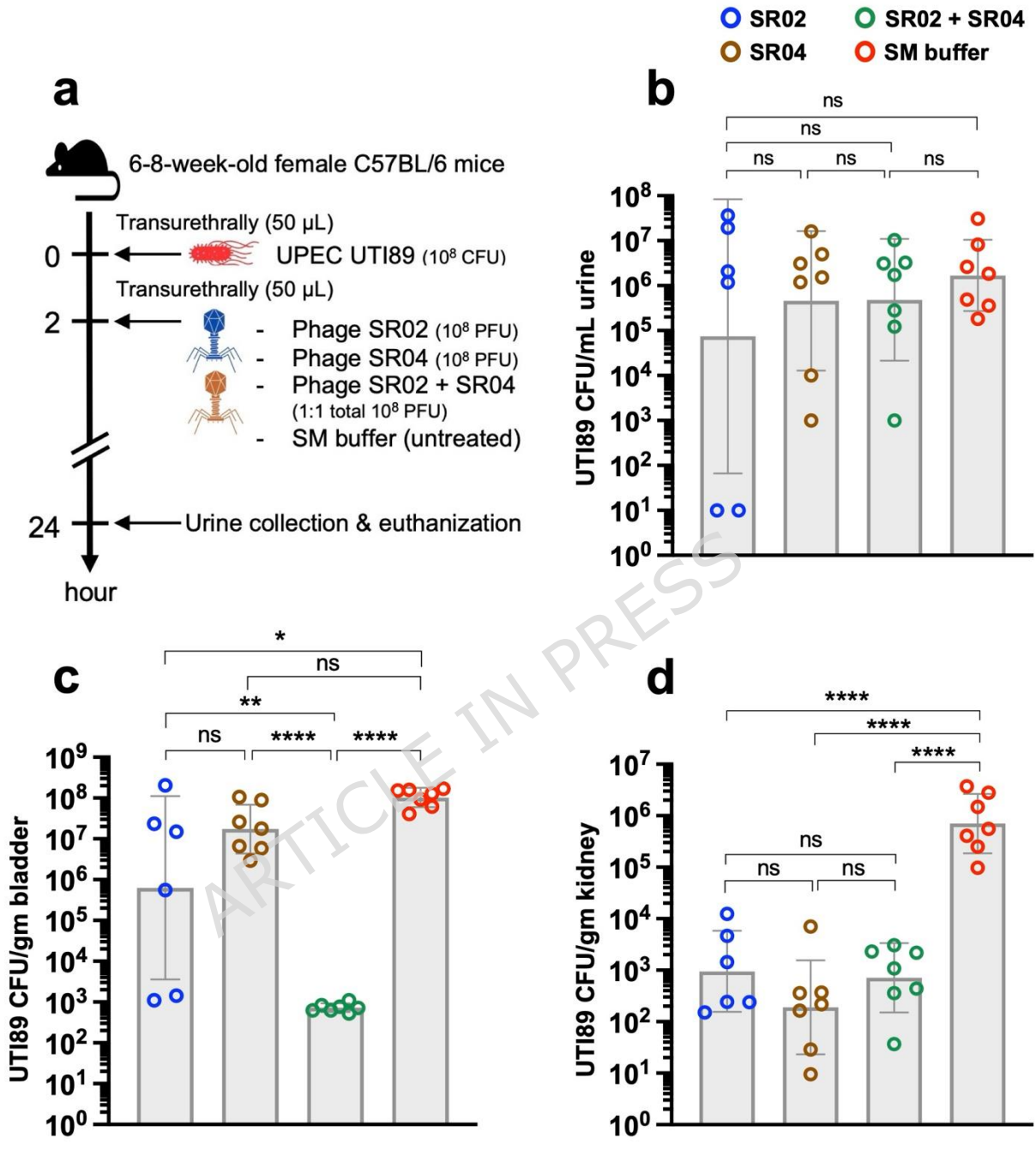
a
6-8-week-old female C57BL/6 mice
Transurethrally (50 µL)
0
UPEC UTI89 (10⁸ CFU)
Transurethrally (50 µL)
2
- Phage SR02 (10⁸ PFU)
- Phage SR04 (10⁸ PFU)
- Phage SR02 + SR04 (1:1 total 10⁸ PFU)
- SM buffer (untreated)
24
Urine collection & euthanization
hour
b
SR02
SR02 + SR04
SR04
SM buffer
UTI89 CFU/mL urine
c
UTI89 CFU/gm bladder
d
UTI89 CFU/gm kidney
ns
*
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Figure 2

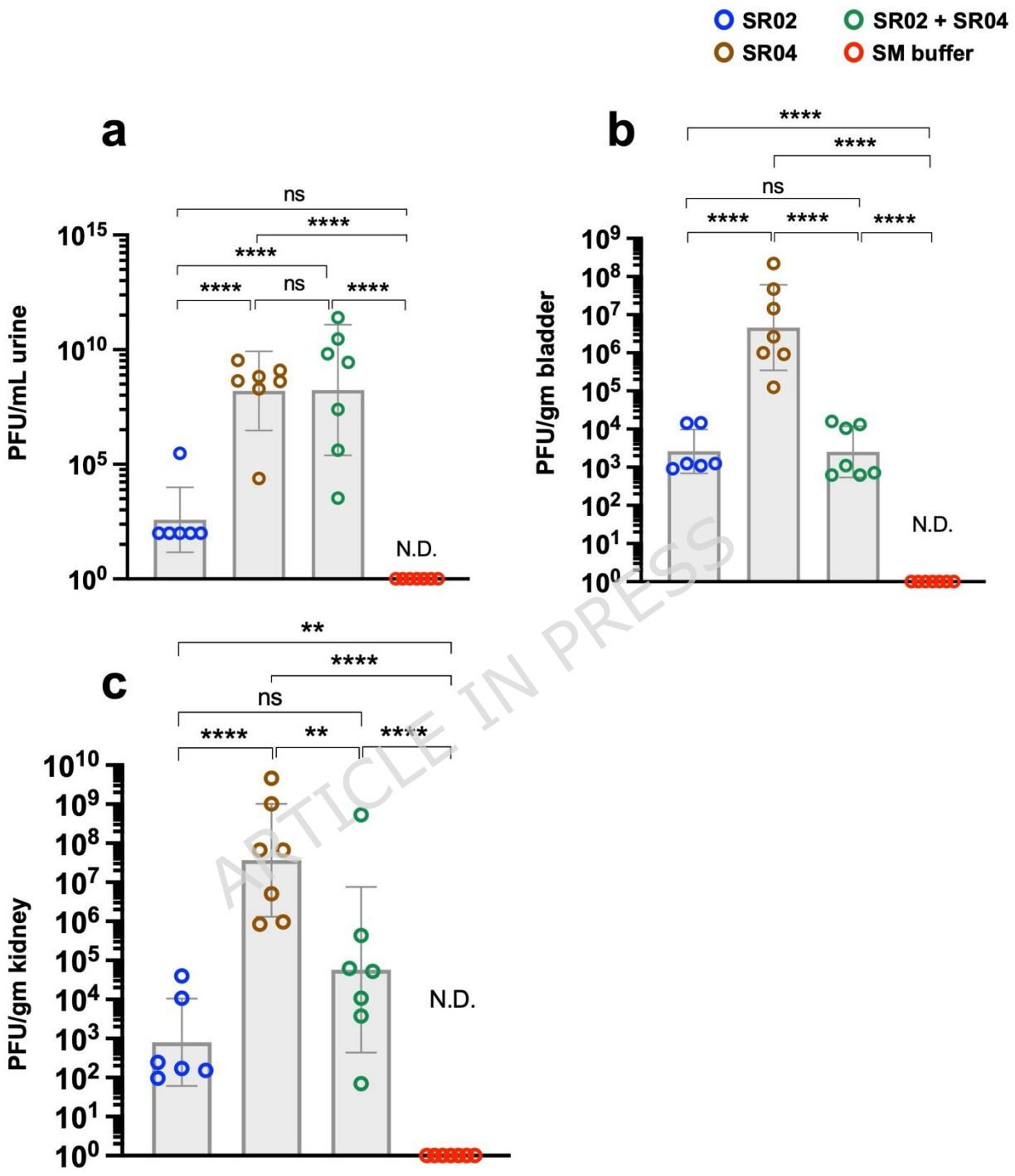

Figure 3

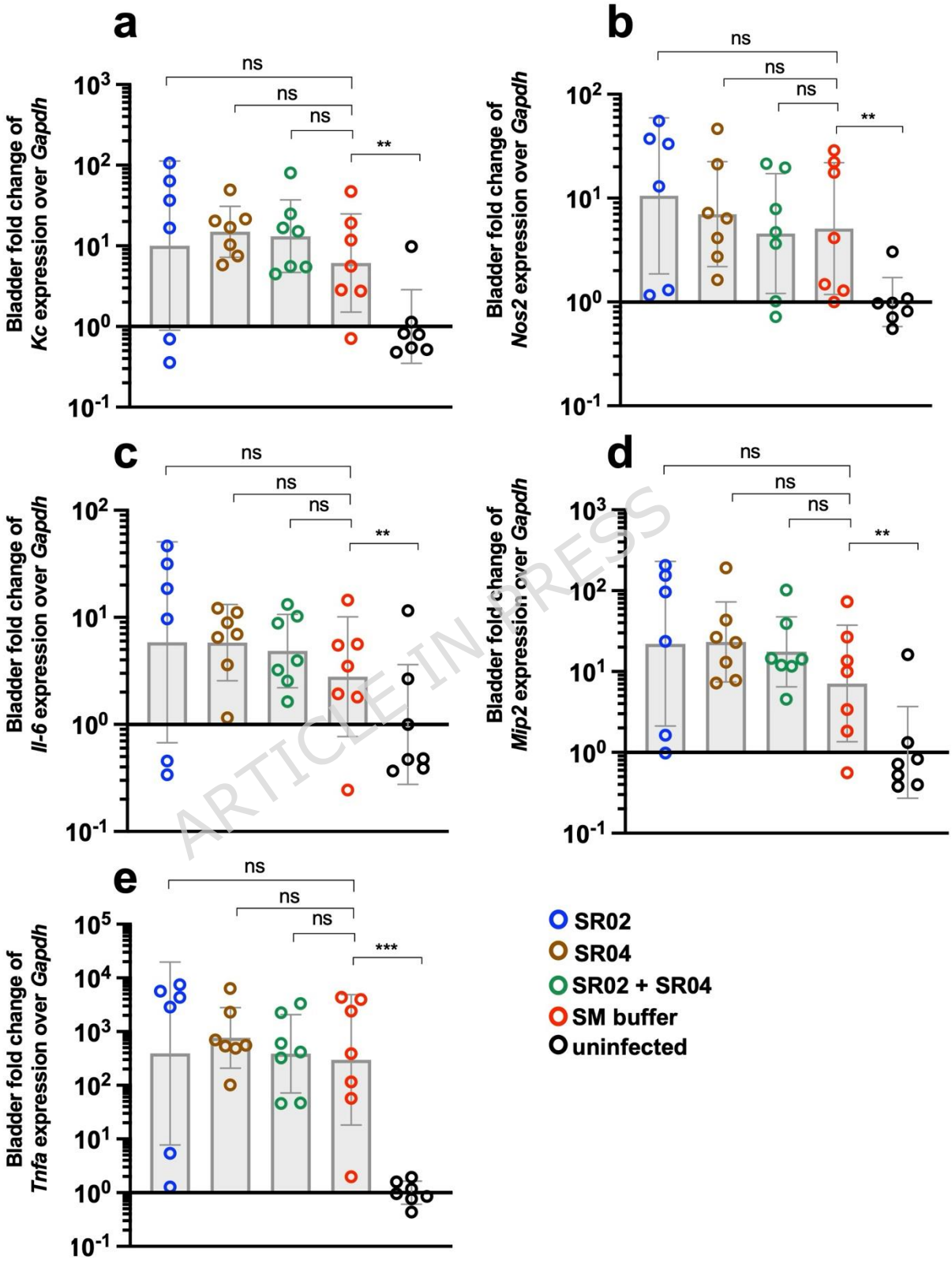
a
b
c
d
e
Bladder fold change of Kc expression over Gapdh
Bladder fold change of Nos2 expression over Gapdh
Bladder fold change of Il-6 expression over Gapdh
Bladder fold change of Mip2 expression over Gapdh
Bladder fold change of Tnfa expression over Gapdh
ns
**
***
SR02
SR04
SR02 + SR04
SM buffer
uninfected

Figure 4

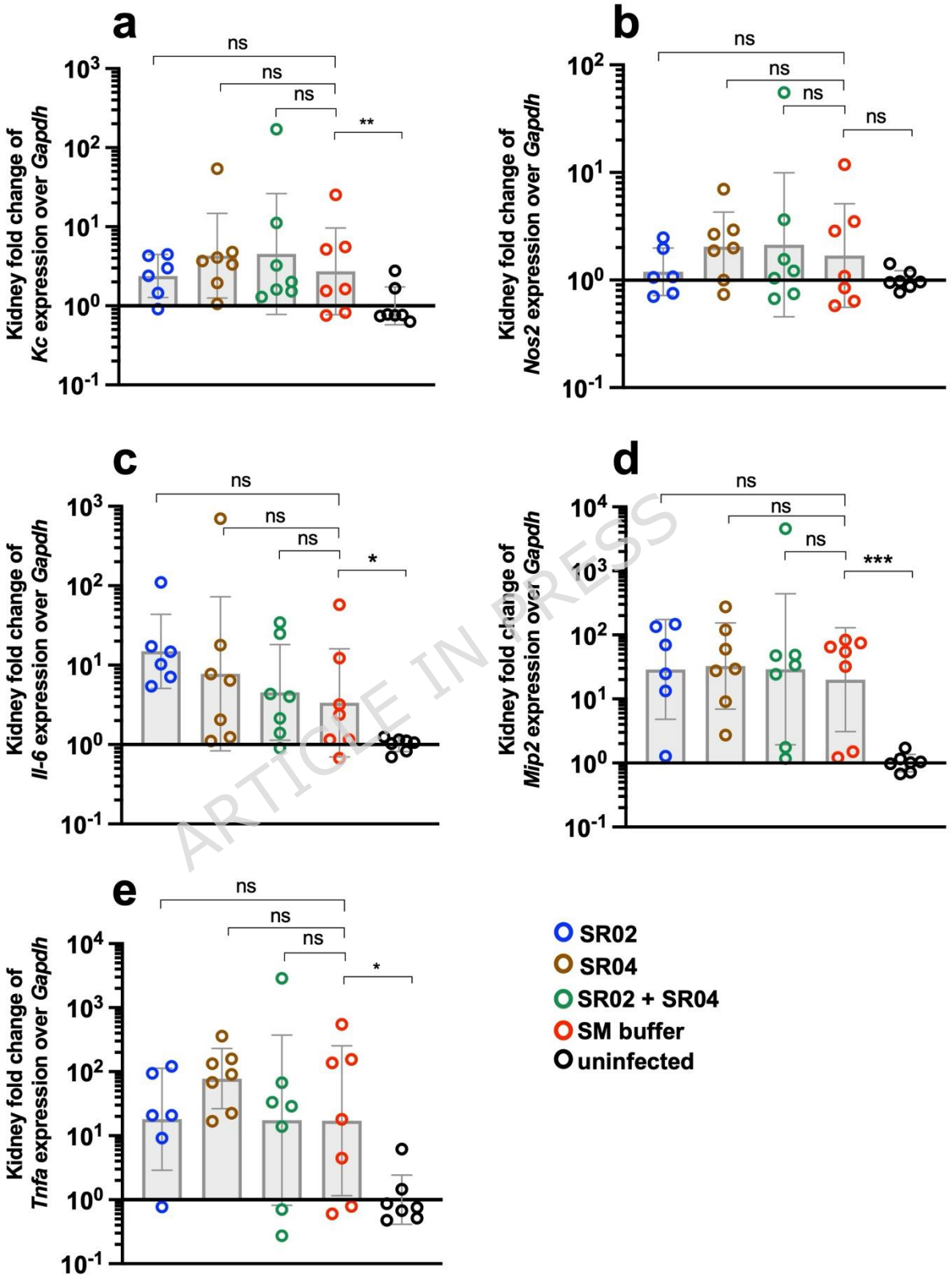

Figure 5

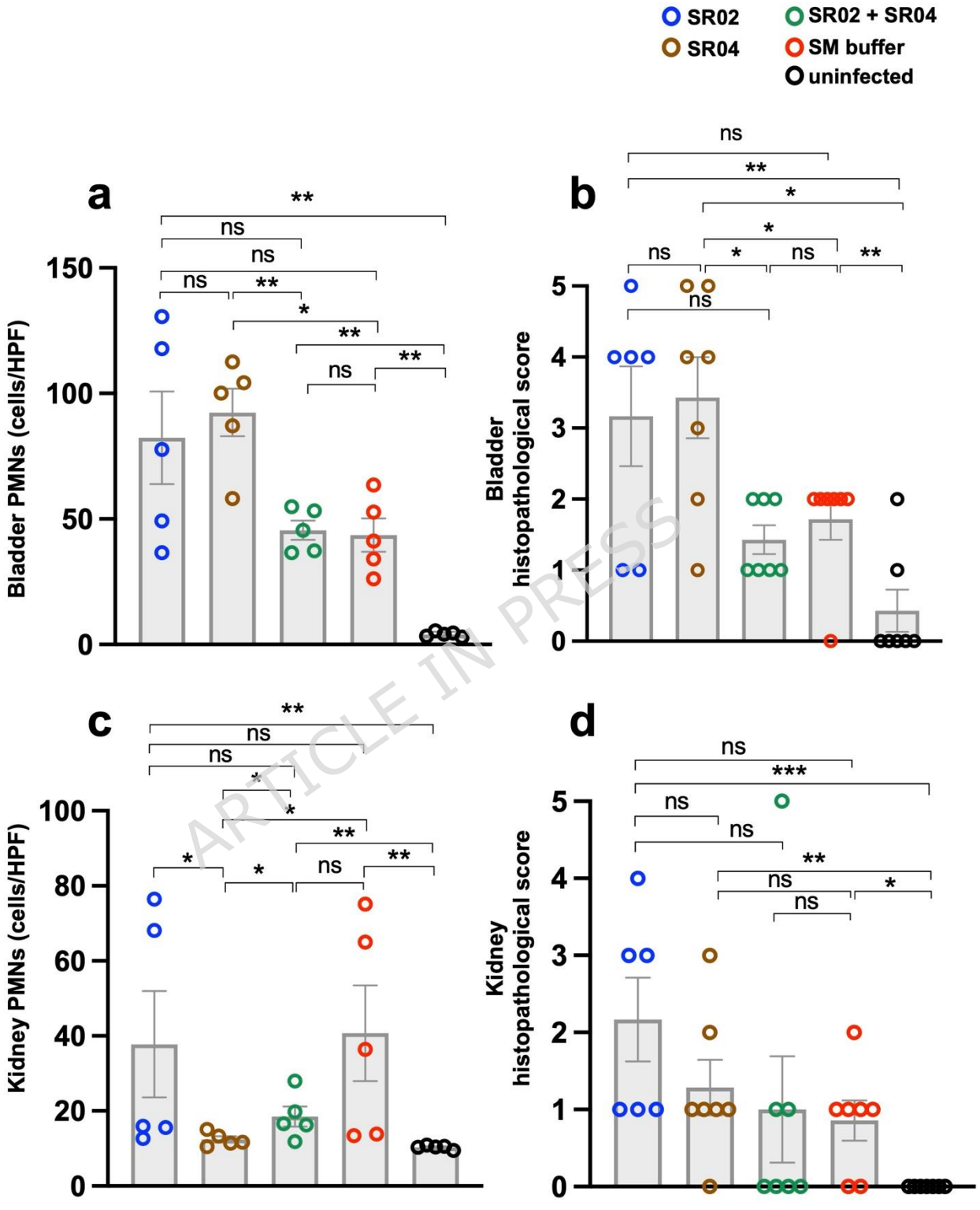

Figure 6

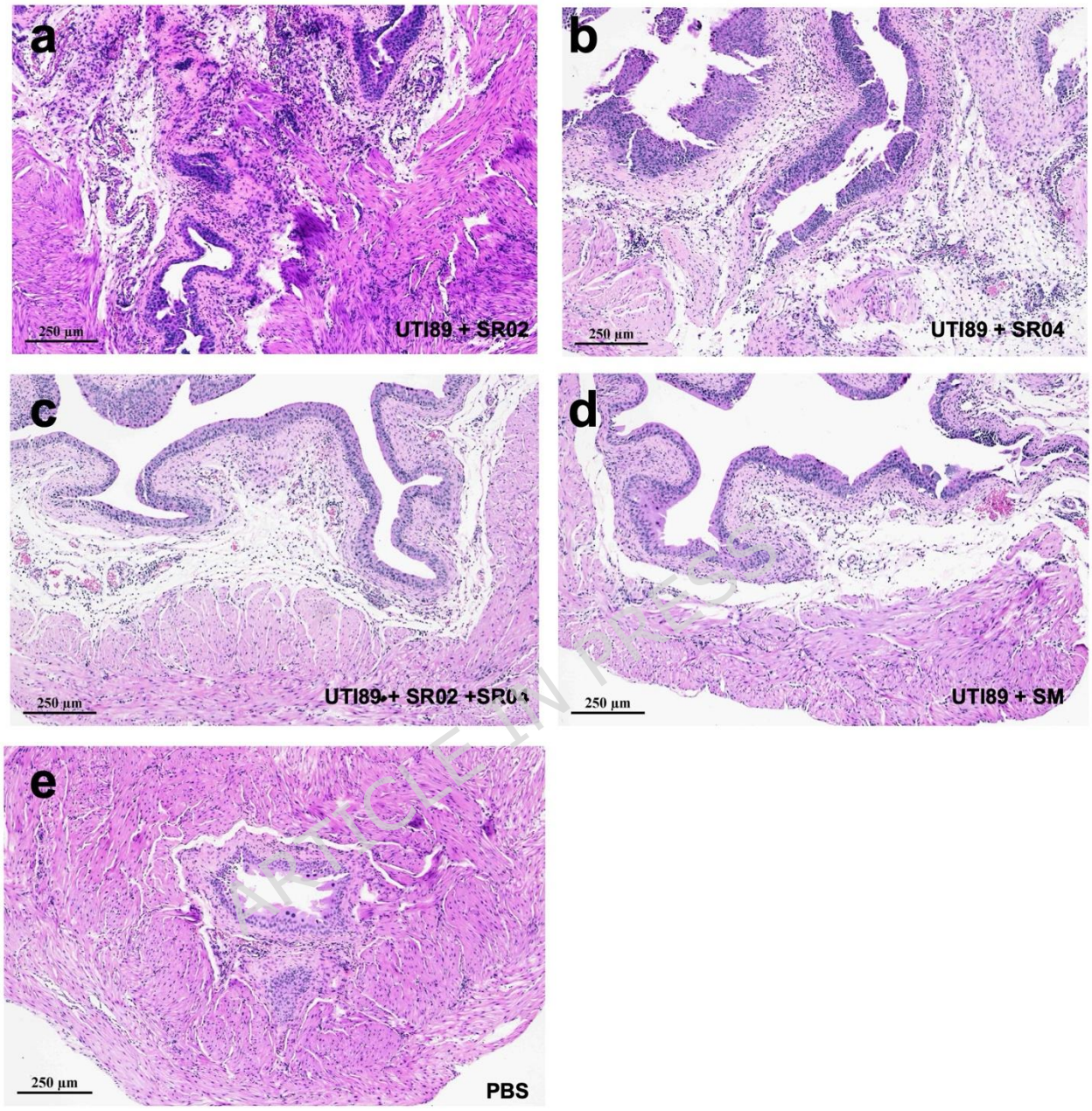

Figure 7

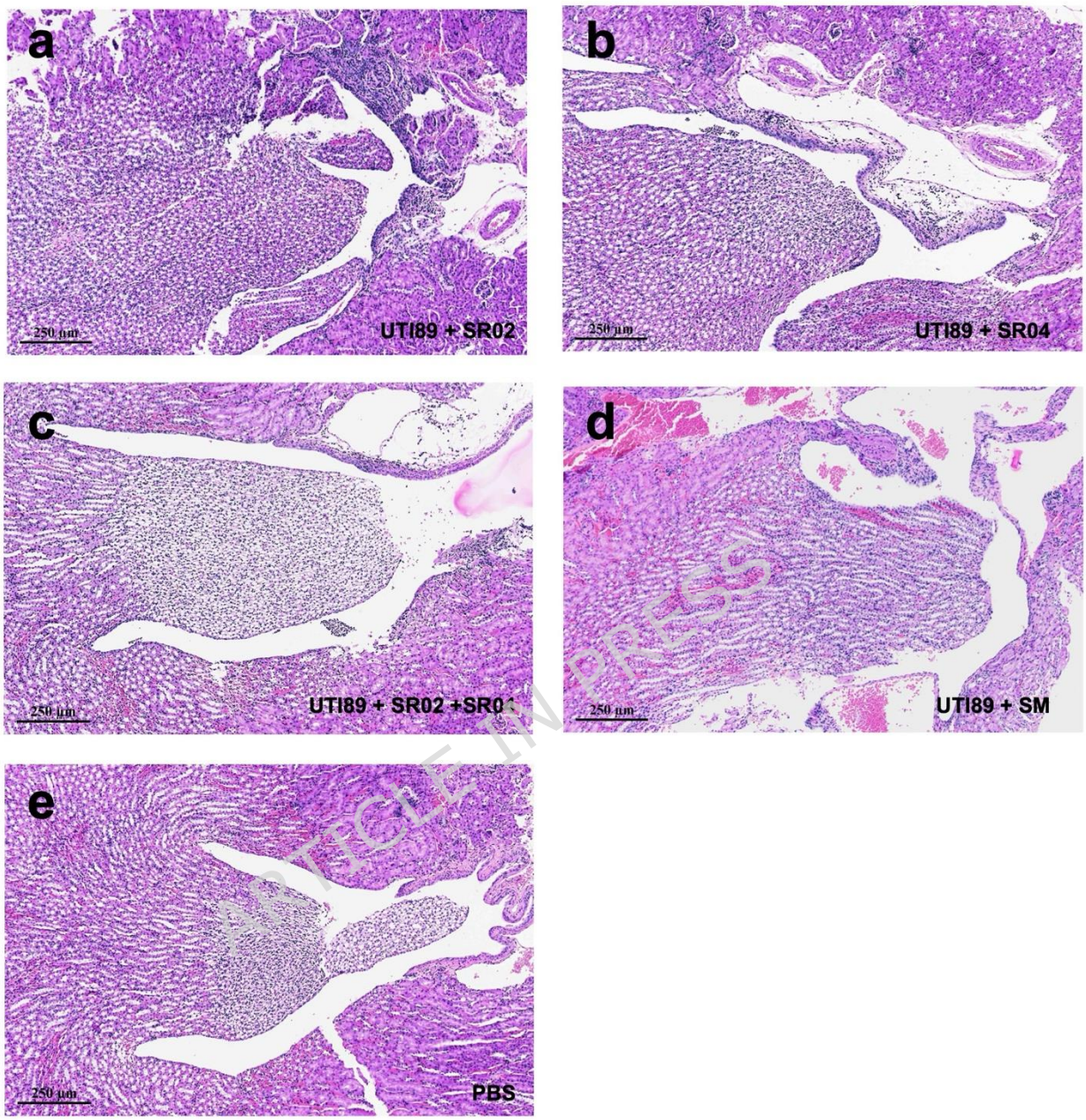

Figure 8