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A novel gene *R049* identified in uropathogenic *Escherichia coli* provides partial protection in mice from colonization

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Uropathogenic *Escherichia coli* (UPEC) is the most common causative organism of human urinary tract infection (UTI). Several UPEC virulence factors have been identified, but more are yet to be found. We previously identified a novel 789-bp-long DNA fragment (named R049) in UPEC strain 132 using a suppressive subtractive hybridization technique. In the present study, we used genome walking to elongate the sequence of this fragment to obtain the whole gene sequence and examined the role of this gene product in generating protective immunity. Through bioinformatic analysis, we predicted that this gene is a 1311-bp open reading frame (ORF), which we designated ORF_{R049} (GenBank accession No.: EF488001). We further constructed a prokaryotic expression system to express full recombinant R049 protein and isolated and purified the protein through IPTG induction and nickel affinity chromatography. Using mouse immunosera generated by the purified protein, we confirmed the natural expression and outer membrane localization of the protein in wild-type strain UPEC132 by Western blotting. To test the potential of this protein as a vaccine candidate, we immunized mice with the recombinant protein before challenging them with UPEC132 through the urinary tract. The results showed significantly reduced bacterial colonization in the urine and kidneys of the immunization group compared with the control group. However, the degree of renal pathological damage was not significantly improved in the immunized mice. Our study has identified a novel gene of UPEC which can generate protective immunity against UTI. This novel gene provides a promising new vaccine candidate.

uropathogenic *Escherichia coli*, genome walking, cloning and expression, immunoprotection

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Uropathogenic *Escherichia coli* (UPEC) is the most common etiologic agent of urinary tract infection (UTI) [1,2]. UPEC strains are distinct from most diarrheagenic or commensal *E. coli* isolates because they produce a number of UTI-associated virulence factors that permit their successful colonization in the urinary tract. A range of putative and established virulence genes have been identified including fimbrial adhesins (e.g., type 1 and P fimbriae), toxins (e.g., cytotoxic necrotizing factor 1, hemolysin and secreted autotransporter toxin), host defense avoidance structures (e.g., capsule), and multiple iron acquisition systems (e.g., aerobactin, enterobactin and yersiniabactin) [3,4]. However, many *E. coli* strains isolated from patients with UTI either have none or just one of these known virulence factors. Notably, a comparative genomics study demonstrated that UPEC strains possess genomes that are 20% larger than that of the nonpathogenic *E. coli* strain K-12 [5]. Thus, it is reasonable to predict that more pathogenic genes are yet to be discovered which may be important in UTI pathogenesis.

The discovery of new UTI-associated genes is important in studying the pathogenic mechanisms of UPEC [6,7]. In addition to the virulence factors described above, a series of new virulence genes have been identified in UPEC strains

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in recent years, which include the heat-resistant agglutinin gene *hra* [8], vacuolating cytotoxin gene *sat* [9] and fimbrial gene *auf* [10]. These findings provide further insights into the interaction between UPEC and host cells and host defense mechanisms.

In previous work, we used suppressive subtractive hybridization to search for new virulence gene candidates for UTI pathogenesis. The subtraction was performed between the tester strain, UPEC132 (a pyelonephritis-causing *E. coli* strain), and the driver strain, MG1655 (a nonpathogenic *E. coli* strain). After subtraction, 37 tester-specific fragments were obtained and one of them, a 789-bp-long fragment designated *R049* (GenBank accession No.: EF488001), showed no homology to sequences in the GenBank database [11]. In the present study, we used genome walking to obtain the whole sequence of this new gene and validated its expression in strain UPEC132 by SDS-PAGE and Western blotting analysis. Furthermore, we assessed the immunoprotective effects of recombinant R049 protein in a mouse model of UTI.

1 Materials and methods

1.1 Bacterial strains

UPEC132 was isolated from the urine of a patient with acute pyelonephritis in Tianjin, China [12]. A total of 20 UPEC strains and 40 fecal isolates of *E. coli* were used to screen fragment R049. The UPEC strains included 16 isolates from patients with pyelonephritis and 4 isolates from patients with cystitis in the General Hospital of Tianjin Medical University (Tianjin, China). The fecal isolates were collected from adults without UTI in the Tianjin Centers for Disease Control and Prevention (Tianjin, China). *E. coli* Top10 and BL21 (DE3) were used as host strains for the transformation of plasmids. These *E. coli* strains were routinely grown at 37°C in Luria-Bertani (LB) broth or on LB agar. When appropriate, ampicillin was added to the growth media at a concentration of 50 μg/mL.

1.2 PCR amplification of fragment R049 in *E. coli* isolates

Fragment R049 was amplified from the chromosomal DNA of UTI and fecal isolates by PCR with primers R049-P1 (forward, 5′-CAGCCAGGAGATTACAAC-3′) and R049-P2 (reverse, 5′-ACTGAAATAGCCATAACG-3′). The predicted amplification product was 631 bp in size (from base 11–641). Amplification consisted of denaturation (94°C, 5 min), followed by 25 cycles of denaturation (94°C, 1 min), annealing (48.6°C, 1 min), extension (72°C, 1.5 min), and a final extension step (72°C, 7 min).

1.3 Genome walking and sequence analysis

The regions flanking fragment R049 in UPEC132 were obtained by PCR using a commercial Genome Walker kit (Clontech, CA, USA). Briefly, the method involved ligation of adapters to purified uncloned libraries of genomic DNA digested with different restriction enzymes. The adapter primers provided in the kit and 2 sets of gene-specific primers (GSPs) were used to PCR amplify regions upstream and downstream of the tester-specific fragment according to the manufacturer's specifications. For amplifying downstream regions, the primers for the primary PCR were Adaptor Primer 1 (AP1; provided in the kit) and GSP1 (5′-TCTTCATGTGCGTAGAAAAGGCGTTGG-3′). The nested PCR was carried out with primers Nested Adaptor Primer 2 (AP2; provided in the kit) and GSP2 (5′-TGAACCACACTGAACCAACAACACGG-3′), which were designed to confirm the primary PCR products. For amplifying upstream regions, the primers for the primary PCR were AP1 and GSP3 (5′-GTGTACAAGGGCGTGACGTTTTTGAAAG-3′). The primers for the nested PCR were AP2 and GSP4 (5′-GCACAACACCTGGCTGGCATCAGAGAGT-3′). The PCR product was cloned into pMD 18-T vector (Takara, Dalian, China) and transformed into *E. coli* Top10 cells for sequence analysis. Proscan (Ver. 1.7) was used to analyze the DNA sequence for open reading frames (ORFs). Homology searches were performed by comparing the sequences to the public DNA and protein databases using the BLASTN and BLASTX search programs (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov).

1.4 Cloning, expression and purification of recombinant R049 protein

The pET-32a(+) expression system (Novagen, USA) was used to express recombinant protein R049. ORF_{R049} was amplified from genomic DNA of UPEC132 using the following primer pairs: 5′-CCGGAATTCCCTGATGGGGAAATCATCG-3′ and 5′-CCGCTCGAGTTTCTTCAGATGAGTTGTAA-3′. The PCR product was gel purified, cloned into the vector, and transformed into *E. coli* Top10 cells. Recombinant plasmid, designated pET32a-R049, that allowed for the expression of protein R049 fused with the 109 amino acid Trx·Tag thioredoxin protein and the N-terminal His·Tag, was selected. Plasmid pET32a-R049 was transformed into *E. coli* BL21 (DE3) cells. After induction with isopropyl-β-D-thiogalactopyranoside and lysis by ultrasonic waves, the crude recombinant protein was purified by nickel affinity column chromatography (HisTrap™ HP Columns; GE Healthcare, USA) in accord with the manufacturer's instructions. The purified protein was analyzed by SDS-PAGE, renatured by dialysis, and assessed by Bradford's method to detect the concentration.

1.5 R049 antibody production and titer determination

One hundred micrograms of purified recombinant protein

R049 was homogenized with complete Freund's adjuvant (Sigma, MO, USA) and administered subcutaneously to 10-week-old female BALB/c mice. Booster immunizations were given 3 times at 2-week intervals with Freund's incomplete adjuvant. On the 5th day after the last immunization, the mice were bled by cardiac puncture and the sera were separated. Enzyme-linked immunosorbent assay (ELISA) was used to determine the antibody levels to purified recombinant protein R049.

1.6 Purification of membrane fractions from UPEC132

Inner and outer membranes were isolated by sucrose density gradient centrifugation as described previously [13], with minor modifications. LB broth cultures (500 mL) were grown with shaking at 37°C to late log phase, harvested by centrifugation, resuspended in 5 mL of 50 mmol/L Tris-HCl (pH 8.0), and then lysed by two passages through a French press. After the elimination of unbroken cells by centrifugation, the supernatant was loaded onto 55%, 45%, 40%, 35% and 30% discontinuous sucrose gradients and subjected to centrifugation for 2 h at 180000×*g* at 15°C. The crude extract membrane fraction was collected and then separation on a second discontinuous sucrose gradient was performed. After centrifugation for 18 h at 150000×*g* at 15°C, 1 mL fractions were collected by piercing the bottom of the tube. To detect the inner membrane fractions, lactate dehydrogenase activity was measured. The main component of the core polysaccharide of LPS, 2-keto-3-deoxyoctonic acid, was used as a marker of the outer membrane fractions and was detected by thiobarbituric acid colorimetry.

1.7 Immunoblot analysis

Whole-cell lysates of UPEC132 and purified recombinant protein R049 were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. In a separate gel, the inner membrane proteins, outer membrane proteins, and whole-cell lysates of UPEC132 were electrophoresed under denaturing conditions and transferred to PVDF membrane. The PVDF membranes were blocked overnight in 5% nonfat dry milk in PBS containing 0.5% Tween 20 at 4°C, and were then probed with primary antibody (anti-recombinant protein R049 sera) and horseradish peroxidase-conjugated anti-mouse sera as the secondary antibody. The membranes were thoroughly washed and developed with a solution of 4-chloro-1-naphthol in methanol.

1.8 Immunization protocols and challenge experiment

Thirty-two 10-week-old female BALB/c mice were randomly divided into 4 groups (groups A–D). The mice of group A were immunized subcutaneously with 100 μg of purified recombinant protein R049 according to the protocol described above. The mice of group B were injected subcutaneously with 100 μL of Freund's complete and Freund's incomplete adjuvant alone. On the 5th day after the last immunization, a 50-μL blood sample was drawn from the medial canthus of each mouse in groups A, B, and C, and the sera were separated and the titer was detected by ELISA. Groups A, B, and C were challenged with 10^8 colony forming units (CFU) of UPEC132 in 100 μL by intraurethral catheterization as described previously [14]. One hundred microliters of sterile phosphate buffered saline (PBS) (0.01 mol/L, pH 7.4), which served as a negative control, was administered to the mice of group D by urethral catheterization.

1.9 Quantitation of infection

Animals were euthanized on the sixth day after infection by cervical dislocation. Ten microliters of urine from each mouse were collected. Urine samples were inoculated onto 0.5 cm^2 areas of LB plates and incubated for 18–24 h at 37°C. Specimens were assessed for relative colonization density (RCD) per 0.5 cm^2 areas by the following grading criteria [15]: 5+ (confluent growth, >90% of the area); 4+ (not confluent growth but too numerous to count); 3+ (>20 CFU); 2+ (≤20 CFU); 1+ (≤3 CFU); and 0 (no growth).

Upon euthanization, each kidney was removed aseptically and halved. The cut surface was streaked onto LB agar and the plates were incubated for 18–24 h at 37°C. The mean number of colonies for two kidneys on a 2 cm^2 areas impression streak were assessed as described above. The urine and renal isolates were identified by routine methods and PCR amplification of the *papC* gene encoding the P fimbriae of UPEC strains was performed as described previously [16].

1.10 Histological examination

The kidneys were preserved in 10% formalin and sectioned through their central region, which contains the papilla, medulla, and cortex. Sections were stained with hematoxylin and eosin, and evaluated in a blind manner by a pathologist. The severity of renal pathology was graded with a scoring system in which 0, 1+, 2+, and 3+ indicated normal, mild, moderate, and severe pyelitis, respectively; 4+, 5+, and 6+ indicated mild, moderate, and severe pyelonephritis, respectively [17].

1.11 Statistical analysis

Statistical analysis was performed using SPSS for Windows, version 10.0 (SPSS Inc., USA). The statistical methods used in this study included the chi-square test and analysis of variance (ANOVA), with $P<0.05$ considered

significant for all comparisons.

2 Results

2.1 ORF search and prevalence of fragment R049

A 3498-bp sequence flanking fragment R049 was obtained by genome walking. Proscan analysis of this sequence revealed the presence of a probable ORF (1311 bp), designated ORF_{R049}. The nucleotide sequence of ORF_{R049} was submitted to the public DNA and protein databases for homology searches. The alignment results revealed that no known genes or proteins shared similarity with the submitted sequence. The complete sequence of ORF_{R049} was submitted to the GenBank database to replace the previously submitted partial sequence of fragment R049, and the accession number was the same as the original submission, EF488001.

PCR of a 631-bp region of fragment R049 was performed to determine whether fragment R049 was present in other strains of UPEC and intestinal *E. coli* isolates. Fragment R049 was found to be present in 8 of the 20 (40%) UPEC strains tested, but in only 3 of the 40 (7.5%) fecal isolates tested ($P<0.01$), indicating that fragment R049 was significantly more prevalent among UPEC strains than fecal *E. coli* strains [18]. Therefore, fragment R049 had a higher correlation with UTI-causing strains and may be part of a potential UTI-associated gene.

2.2 Expression and purification of recombinant protein R049

ORF_{R049} was amplified and cloned into the pET-32a(+) expression vector, and was then transformed into *E. coli* BL21 (DE3) cells. *E. coli* BL21 (DE3)/pET32a-R049 cells expressed a protein of approximately 66.9 kD, whereas *E. coli* BL21 (DE3) cells containing the control expression vector did not express this protein (Figure 1). The observed size of the recombinant protein was larger than the predicated mass of the gene product of ORF_{R049} (47.7 kD), likely because of the presence of a 109 amino acid Trx·Tag thioredoxin protein and an N-terminal His·Tag. After nickel affinity purification, recombinant protein R049 showed a single band on SDS-PAGE, and the concentration was determined to be 2 mg/mL by Bradford's method.

2.3 UPEC132 expression of gene *R049*

To confirm the expression of gene *R049* in wild-type strain UPEC132, antisera to the purified recombinant protein R049 were generated by immunizing BALB/c mice. The titer of antisera was ⩾1∶12800, as assessed by ELISA. Whole-cell lysates of UPEC132 were examined by Western blot analysis using our anti-recombinant protein R049 antisera. Purified recombinant protein R049 was loaded onto the gel as a positive control. A UPEC132 protein, of approximately 47.0 kD, was detected to react with the antisera, and the observed size was consistent with the predicated mass of the natural gene product of ORF_{R049} (47.7 kD, lane 3 in Figure 2(b)) and was smaller than the recombinant fusion protein (66.9 kD, lane 2 in Figure 2(b)). These results indicated that gene *R049* indeed exists in the genomic DNA of UPEC132, confirming the prediction of ORF_{R049} by bioinformatic analysis.

To determine the cellular location of protein R049, the inner and outer membrane fractions of UPEC132 were separately detected with anti-recombinant protein R049 antisera. A 47.0-kD protein in both the whole-cell lysates and the outer membrane fractions reacted with the antisera, and the observed size was consistent with the predicated mass of ORF_{R049} (Figure 3(b)). This indicated that R049 was an outer membrane protein of UPEC132.

2.4 Assessment of the immunoprotection of recombinant protein R049 in a mouse model of UTI

The protein R049-immunized (group A), Freund's adjuvant-immunized (group B), and unimmunized (group C)

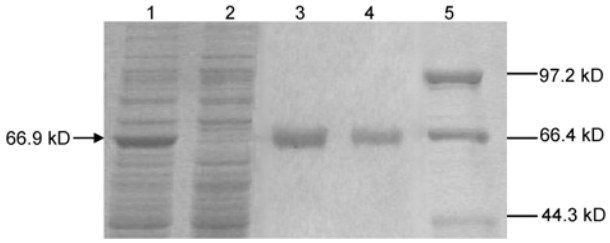

Figure 1 Expression and purification of induced recombinant protein analyzed by SDS-PAGE. The recombinant strain *E. coli* BL21 (DE3)/pET32a-R049 overexpressed a 66.9-kD protein (the arrow indicates recombinant protein R049 in lane 1), which was not expressed in *E. coli* BL21 (DE3) cells containing the control expression vector (lane 2). After affinity chromatography (lane 3) and dialysis (lane 4), nearly all other proteins were removed. The molecular weight standards are shown in lane 5.

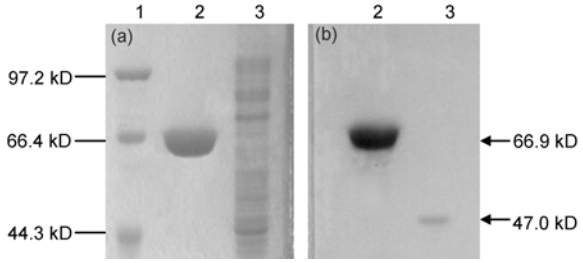

Figure 2 SDS-PAGE (a) and Western blot analysis (b) of recombinant protein R049 and the total bacterial proteins of UPEC132. (a) 1, molecular weight standards; 2, recombinant protein R049; 3, total bacterial proteins of UPEC132. (b) Lane numbers correspond to those in panel (a). The 66.9-kD recombinant protein, which served as a positive control, reacted with the antisera. A 47.0-kD protein of the total bacterial proteins of UPEC132 also reacted with the antisera, and the observed size was consistent with the predicated mass of ORF_{R049}.

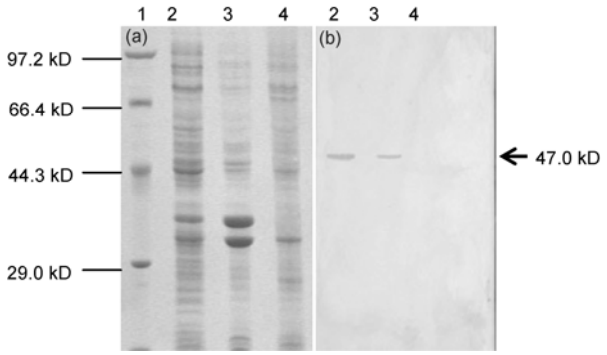

Figure 3 Cellular localization of protein R049. (a) The whole-cell lysates, outer membrane proteins, and inner membrane proteins of UPEC132 were analyzed by SDS-PAGE. 1, molecular weight standards; 2, total bacterial proteins; 3, outer membrane proteins; 4, inner membrane proteins. (b) The proteins described above reacted with the antisera raised against recombinant protein R049. Lane numbers correspond to those in panel (a). A 47.0-kD protein of the whole-cell lysates and outer membrane proteins reacted with the antisera, and the observed size was consistent with the predicated mass of ORF_{R049}.

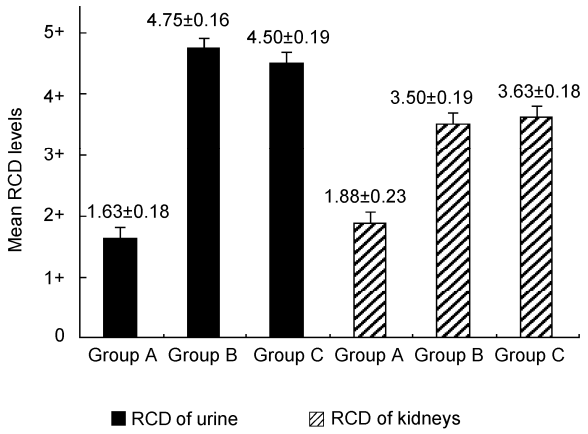

Figure 4 Comparison of the mean relative colonization density (RCD) levels of the urine and kidneys between the immunized and unimmunized mice following challenge infection. RCD levels of group A were significantly lower than those of group C in the urine (P=0.0001) and kidneys (P=0.0001). The bacterial loads between groups B and C showed no statistical differences (P=0.334 for urine comparison; P=0.664 for kidney comparison). Data are shown as the mean ± standard error.

BALB/c mice were transurethrally inoculated with 10^8 CFU of UPEC132. The mice in group D were injected with sterile PBS by urethral catheterization as a negative control. Mice were euthanized on the sixth day after infection, and the urine and kidneys were aseptically collected to determine the levels of bacterial colonization based on the RCD (Figure 4).

Culture of the urine samples and kidney tissues of group D (sterile PBS-treated mice) were both negative, whereas bacterial loads were observed in the Freund's adjuvant control (group B) and unimmunized challenged mice (group C). The RCD levels of the urine and kidneys of the mice in group C were 4+ to 5+ and 3+ to 4+, respectively, which were similar to the levels of group B (P=0.334 for the urine comparison; P=0.664 for the kidney comparison) (Figure 4). In contrast, the RCD levels of the urine and kidneys of the immunized mice (group A) were 1+ to 2+ and 1+ to 3+, respectively (Figure 4), which were significantly lower than those of group C (P=0.0001 for the urine and kidney comparisons).

The bacterial isolates from the urine and kidneys of group A, B, and C mice were all *E. coli* strains according to the results of routine bacterial identification and were further classified as UPEC132 strains based on *papC* gene amplification. The titers of antisera against the recombinant protein R049 of group A, B, and C were 1∶6400–1∶12800, <1∶100 and <1∶100, respectively, as assessed by ELISA. These results suggested that protein R049 exerts an immunoprotective effect against UPEC infection.

2.5 Renal histological examination

The kidney tissues of group D mice, included as a negative control, showed no obvious histopathological changes. In contrast, mice in groups A, B and C exhibited moderate to severe pyelonephritis after challenge with UPEC132, showing angiectasia and hyperemia, interstitial edema and neutrophil infiltrate in renal parenchyma and renal interstitium, as well as necrosis and exfoliation of the pelvis mucosa (Figure 5). Unlike the significant difference in bacterial loads between the immunized and unimmunized groups, the mean renal pathological scores of groups A and C were similar (5.38±0.52 vs. 5.63±0.52, P=0.35). This indicated that immunization with recombinant protein R049 was unable to block the renal histopathological changes caused by UPEC132 infection, although it was able to reduce bacterial colonization in the kidneys.

3 Discussion

The difference between bacterial loads and kidney histopathologic scores identified in this study may be related to the nature of UPEC, mainly existing as intracellular bacteria in the urinary tract. In previous work, we used confocal microscopy to demonstrate that UPEC132 is an invasive uropathogen which can be internalized by human bladder epithelial cells [19]. A similar finding was reported for *E. coli* cystitis caused by isolate NU14 [20,21], suggesting that UPEC strains are intracellular bacteria. The invasion of host cells can allow pathogens to evade host defenses and ease their dissemination both within and across cellular barriers [22]. Therefore, even low numbers of UPEC132 may invade kidney epithelial cells, thus initiating signaling cascades, such as the recruitment of toll-like receptor 4 [23], the production of cytokines like interleukin (IL)-6 and IL-8, and neutrophil chemotaxis [24,25], to cause inflammation, and finally pathological damage. In this case, although immunization can significantly reduce the bacterial load, the level

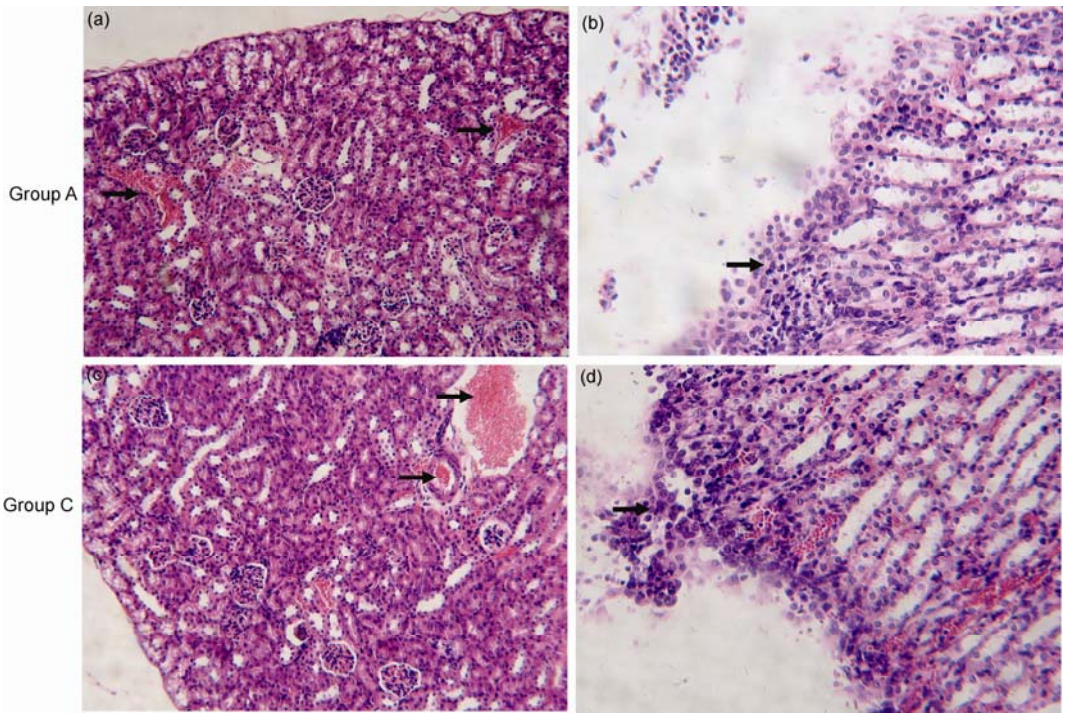

Figure 5 Representative photomicrographs of the kidneys from groups A and C mice showing severe pyelonephritis. The histopathological changes were characterized by angiectasia and hyperemia in the renal parenchyma and interstitium (indicated by arrows in (a) and (c)), and monocyte and neutrophil infiltration in the pelvis (indicated by arrows in (b) and (d)). Aggregates of inflammatory cells within the pelvic lumen are also noted (left upper corner of (b)).

of the bacteria may be still sufficient to cause tissue damage. In other research, we used a cDNA microarray to identify gene expression differences between human uroepithelial cells infected with UPEC132 and uninfected cells, and found differentially expressed genes involved in cell growth and proliferation (e.g., Jun, Fos, IER2, IER3, and EGR1), cytokines (e.g., IL-6, IL-8 and TNF), and apoptotic responses (data unpublished). These findings revealed that signal pathways indeed participate in the interaction between UPEC and host cells.

However, it should be noted that the protection study was only performed at one time point, which was relatively early in infection, and only one infection dose was tested. It is unclear whether immunization with R049 protein will promote the resolution of pathological changes and bacterial clearance in later stages of infection or following challenge with a different infection dose. It is also unclear if using other vaccination techniques, such as DNA vaccination which is more efficient for intracellular infections, will improve the protective immunity and minimize pathological reactions.

Employing an immunization approach to evaluate the potential role of a newly identified gene product has been instrumental in the search for new vaccine candidates. Several UPEC virulence-associated factors, such as FimH, the type 1 fimbrial adhesin [26], PapG, the P fimbrial adhesin [27], and hemolysin [28], have been tested as vaccine targets. In recent years, Hagan et al. used an immunoproteomic approach to identify potential vaccine targets in a pyelonephritis strain of *E. coli* CFT073, and identified 23 antigenic proteins that elicited an immune response during infection [29]. We therefore suggest that the antigenic gene product *R049*, which has been shown to reduce the bacterial load following immunization, is a new vaccine candidate. The discrepancy observed between the effect of immunization on the bacterial load and the pathological changes also suggests potential using this gene product to understand the mechanisms of UTI pathogenesis. Further studies are now required to fully elucidate the function of this gene product in UPEC biology and pathogenicity.

In conclusion, we have identified a novel gene *R049* in UPEC strain 132. The immunoprotective capability of the recombinant protein suggests that the product of gene *R049* is a promising vaccine candidate to prevent and control of UTIs. The molecular mechanism behind this immunoprotection and the function of protein R049 in bacterial pathogenicity require further investigation.

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