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Short Communication

Zoonotic Potential of Canine Uropathogenic *Escherichia coli* Isolated from Yangzhou, China

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ABSTRACT

Uropathogenic *Escherichia coli* (UPEC) is a common pathogen of urinary tract infection. To investigate its characteristics and explore the interaction between UPEC and human urinary bladder cancer T24 cells, 7 canine UPEC strains were isolated from dogs in Yangzhou, China. The adhesion-encoding genes (*iha*, *fimH*, *papA*, *papC*, *papG allele I*, *papG allele I'*, *papG allele II*, *papG allele III*, *focA*, *focG*, *sfaS*), virulence-associated genes (*sat*, *cdtI*, *cnf1*, *hlyD*), iron uptake system encoding genes (*iroN*, *iut*, *ireA*) were investigated, and the ability of biofilm formation was examined. In addition, UPEC in invading T24 cells, cytotoxicity, immune response and synthesis of proinflammatory cytokines were discussed. The results showed that *fimH* was 100% detected, followed by *iut*, *iroN*, and *focA*, with the detection rates of 71.4, 57.1 and 57.1% respectively; while *papG allele I*, *papG allele I'*, *papG allele II*, *papG allele III*, *cdtI*, *sat*, *sfaS*, *iha* and *sat* were not detected. Most of the isolated UPEC strains have a strong virulence on T24 cells and could induce strong immune response. Taking these data together, canine UPEC strain may not be a canine specific pathogen, but has a certain potential for zoonosis.

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ZG and YY conceived and designed the experiments. ZX, WP and WY performed experiments. YY and ZX wrote this paper. All authors have approved the paper for publication.

Key words
Uropathogenic *Escherichia coli* (UPEC), Urinary tract infections, Canine, T24 cells

Urinary tract infections (UTIs) is a common disease in lower urinary tract syndrome, which seriously endangers the health of people and animals. Uropathogenic *Escherichia coli* (UPEC) is a common pathogen which causes more than 80% of UTI. Previous investigations showed that the characteristic related virulence of human UPEC was detected in canine UPEC strains, including P fimbriae, hemolysin and so on (Buberg *et al*., 2021; Kathayat *et al*., 2021; De Souza *et al*., 2019). Therefore, canine UPEC strains may be infected by pets and pose a pathogenic threat to humans.

The pathogenesis of UPEC includes the colonization of UPEC in the urethral region, infection of bladder and growth and reproduction in urine, adhesins such as type 1 fimbriae (T1F) and P fimbriae adhering to the bladder surface and interaction with the epithelial defense system, biofilm formation, invasion of epithelial cells, cause host tissue damage, and increasing the risk of bacteremia or sepsis (Mitsumori *et al*., 1998; Johnson and Stell, 2000; Subashchandrabose *et al*., 2014). In China, researches on the epidemiology and pathogenesis of UPEC mainly focus on human UPEC, canine UPEC and its potential zoonotic risk remains to be studied.

The purpose of this study was to collect canine UPEC strains from Yangzhou, and to detect the virulence genes; biofilm formation, cytotoxicity, invasion and immune response of human urinary bladder cancer T24 cells, and comprehensively explore the possibility of zoonosis of canine UPEC strains. It lays a foundation for further study on the pathogenic mechanism and preventive measures of UTI.

Materials and methods

The T24 cells (Bibangbio, Yangzhou, China) were cultured in RPMI 1640 medium containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO_2 (Nam, 2013). DH5α was used as control strain (C), and grown in LB broth and on LB agar plates (Hopebio, Qingdao, China) at 37˚C.

From May 2017 to December 2018, total 80 urine samples were collected through sterile puncture and sterile catheterization from dogs with UTI syndrome in Yangzhou, China. 10 µL of each urine sample was inoculated on sheep blood agar plates (Hopebio, Qingdao, China) at 37°C for 24 h and detected the bacterial count. When the bacterial

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counts ≥ 10^3 colony-forming units (CFU)/mL, strains were inoculated on MacConkey agar (Hopebio, Qingdao, China) and cultured at 37°C for 24 h. Metallic green colonies were presumptively considered as indicators of *E. coli*. The strains were identified by bacterial 16S rDNA PCR Kit (TaKaRa, Dalian, China) (Hu *et al*., 2014), and were preserved as the isolated UPEC strains.

UPEC related virulence genes were detected by PCR using primers (Supplementary Table SI) (Nam, 2013), including adhesion-encoding genes (*iha*, *fimH*, *papA*, *papC*, *papG allele I*, *papG allele I'*, *papG allele II*, *papG allele III*, *focA*, *focG*, *sfaS*), virulence-associated genes (*sat*, *cdtI*, *cnf1*, *hlyD*), iron uptake system encoding genes (*iroN*, *iut*, *ireA*).

For biofilm formation test, the strains were inoculated in LB broth liquid medium at 30ºC in a shaking incubator overnight. The next day, the bacteria solution was adjusted to make OD_{600} = about 1.0, added in 96 well plates for 150 µL per well for inoculation in biofilm induction medium at the ratio of 1:100. After incubated at 30ºC for 48-72 h, each well was rinsed for 3 times by PBS, then 200 µL 2% crystal violet was added for dyeing. After 15 min, each well was gently rinsed with distilled water for 3 times, and then 250 µL 95% ethanol solution was added. The values OD_{600} of each well were measured by a multifunctional microplate reader (Duan *et al*., 2013). All procedures were repeated 3 times, and 6 parallel sets for each strain at a time.

To perform the cytotoxicity assays, T24 cells were inoculated on 96 well plates at 3×10^4 CFU per well and cultured in RPMI 1640 medium (containing 10% fetal bovine serum) at 37 °C with 5% CO_2 for 24 h. Each isolated strain was added at the multiplicity of infection (MOI) of 0.01, and incubated for 4 h. According to the instructions of WST-1 Cell Proliferation Assay Kit (Takara, Dalian, China), 25 µL of cell proliferation reagent was added to the above cell culture well and incubated for 4 h. The OD_{450} was measured with a multifunctional microplate reader. Percent cytotoxicity = $(A_{1640} - Aexp)/A_{DMEM}\times 100$. Aexp: the absorbance of test samples; A_{1640}: the absorbance of negative control in which 1640 medium was added.

To perform the invasion assays, the bacteria were incubated with T24 cells at the MOI of 0.01 for 2 h, then were washed with PBS for 3 times immediately, and antibiotics (50 µg/mL gentamicin) was added to further incubate for 2 h. Then 0.5% Triton X-100 was added for 20 min to release the bacteria that had invaded the cells. After diluting the culture medium, samples were evenly spread on LB medium plate and cultured overnight at 37 °C, then bacteria on plates were counted, and DH5α was used as the negative control.

The isolated bacteria were incubated with T24 cells at the MOI of 1:100 for 2 h, then the total RNA was extracted using Trizol reagent (Takara, Dalian, China) (Yang *et al*., 2013). The primers for pro-inflammatory factors *il-8* and *tnf*-α were listed in Table S1. Gene *gapdh* was used as the housekeeper gene, and SYBR ® Premix Ex Taq II (Takara, Shiga, Japan) was employed for subsequent fluorescence quantitative test. Data were collected by ABI 7500 Real Time System (Applied Biosystems, Foster City, CA, USA), and were processed by $2^{-\triangle\triangle CT}$ method.

Results

Out of the 37 strains of bacteria isolated from urine samples 7 were identified as UPEC, accounting for 18.9 % of the isolates. UPEC usually carries a series of virulence factors, which adhere to host cells and invade the host. The detection rate of *fimH* was 100%, followed by *iut*, *iroN*, and *focA*, with the detection rates of 71.4, 57.1 and 57.1% respectively. The following genes, including *papG allele I*, *papG allele I', papG allele II, papG allele III*, *cdtI, sat*, *sfaS*, *iha* and *sat*, were not detected (Table I). The ability of UPEC strains to biofilm formation was detected by crystal violet staining (Duan *et al*., 2013) (Fig. 1A). Among the isolated strains, 4 strains had strong biofilm forming ability (No. 3, 4, 5 and 7). Compared with the negative control DH5α, the biofilm forming ability was increased by 7.5, 8.6, 8.8 and 7.1 times, respectively (P<0.05).

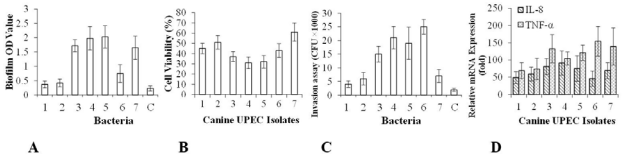

Fig. 1. **A**, Biofilm formation observed at OD_{600}; **B**, cytotoxicity assay WST-1 Kit; **C**, invasion assay the survival CFU of recovered T24 cells, Y-axes indicate averaged CFU values recovered; **D**, transcriptional levels of *il-8* and *tnf*-α. All data were normalized to the housekeeping gene *gapdh*, values indicated the changed folds of expression level of genes; values are means of the results of 3 independent experiments; error bars indicate standard deviations; No. 1-7: 7 canine UPEC strains; C: DH5α.

The results of the invasion assays showed that compared with the control, all the isolated strains showed stronger cell invasive ability, and the number of T24 cells invaded by each UPEC strain increased by 2-13 times (Fig. 1B). The data showed that isolates 1, 2 and 7 were less invasive. At the same time, WST-1 Kit was used to test the cytotoxicity of UPEC to T24 cells. Compared with the control, the number of viable cells in each isolated group decreased by 50-70%, indicating that most of the isolated strains had strong cytotoxicity against T24 cells (Fig. 1C).

To further analyze the changes in expression level of proinflammatory cytokines after the immune response of T24 cells to UPEC strain, the transcription levels of IL-8 and TNF-α cytokines were detected by quantitative

fluorescence PCR (Fig. 1D). Compared with the control, the IL-8 transcription level of UPEC increased 49−91 times, the level of TNF-α transcription increased 69−154 times. Under the stimulation of each strain, the transcription levels of IL-8 and the cytokines TNF-α in T24 cells increased significantly (Yang *et al*., 2013, 2018).

Table I. Distribution of virulence genes in canine UPEC isolates.

Virulence genes	Isolates						
	1	2	3	4	5	6	7
papA	+	-	-	+	-	-	+
papC	-	-	+	-	+	-	+
papG allele I	-	-	-	-	-	-	-
papG allele I	-	-	-	-	-	-	-
papG allele II	-	-	-	-	-	-	-
papG allele III	-	-	-	-	-	-	-
focA	+	-	+	-	+	+	-
focG	-	-	-	-	+	-	-
sfaS	-	-	-	-	-	-	-
iha	-	-	-	-	-	-	-
fimH	+	+	+	+	+	+	+
cdtI	-	-	-	-	-	-	-
sat	-	-	-	-	-	-	-
cnf1	+	-	-	+	+	-	-
hlyD	-	+	-	+	+	-	-
ireA	-	-	-	+	-	-	-
iroN	+	-	+	-	+	+	-
iut	+	-	+	+	+	+	-

+, positive; -, negative

Discussion

E. coli is the main pathogenic factor of UTI in human and livestock, animal derived *E. coli* has zoonotic potential for human infection. This study identified the virulence genes, biofilm forming ability and the biological characteristics of the 7 isolated canine UPEC, and further discussed UPEC in invading T24 cells, cytotoxicity, immune response and synthesis of proinflammatory cytokines.

UPEC usually carries a series of virulence factors, which adhere and invade the host cells (Mulvey *et al*., 1998; Nagamatsu *et al*., 2015; Leatham-Jensen *et al*., 2016). In the process of infection, UPEC can resist the scouring of various body fluids, especially urine, by adhering to the host cells. Therefore, adherence is a crucial aspect of UPEC infection ability. The virulence factors related to UPEC adherence include T1F, P fimbriae and so on. T1F is usually associated with descending urethral infection, and P fimbriae are mainly related to ascending urethral infection. The acquisition of iron is the key condition for UPEC to survive in urinary iron restricted environment.

Biofilm forming ability is one of the virulence determinants of chronic and recurrent bladder infections associated with UPEC (Dhakal *et al*., 2008; Cai *et al*., 2013; Flores-Mireles *et al*., 2015; Spaulding and Hultgren, 2016; Sharma *et al*., 2016; Terlizzi *et al*., 2017). Biofilm is mainly composed of polysaccharides, proteins, nucleic acids and lipids, which constitute a direct environment for bacterial growth. The drug tolerance of bacterial biofilm is usually 10−1000 times that of planktonic bacteria, which is one of the main reasons for the failure of antibiotic treatment. T1F plays an important role in the formation of biofilm. Recent studies have shown that biofilm formation is an important strategy for *E. coli* colonization in the urinary tract (Soto *et al*., 2007; Mabbett *et al*., 2009). Among the isolated strains, 4 strains had strong biofilm forming ability, which was 7.5, 8.6, 8.8 and 7.1 times higher compared with DH5α, that can greatly improve the survival ability in different hosts and effectively improve the pathogenicity.

Epithelial cells are the first line of defense against invading pathogens, they also help to initiate the host's innate and adaptive immune response by producing chemokines, cytokines and antimicrobial peptides (Wood, 2009; Gibson *et al*., 2010; Nakamura *et al*., 2016; Shenagari *et al*., 2018). The immune response to UPEC begins with bacterial invasion of bladder epithelial cells, which induces the production of pro-inflammatory cytokines and recruits neutrophils to the site of infection. A study on mouse cystitis model showed that UPEC can persist in mouse bladder cells to resist antibiotic treatment. Recent studies showed that UPEC can persist in the host epithelial cells for many days and reproduce rapidly in the cells, suggesting that the ability of UPEC to invade epithelial cells plays a key role during UTI (Xia *et al*., 2011). This study found that most of the isolated UPECs had strong ability to invade T24 cells, suggesting the invasive infection of UPEC does not have strict host specificity. Similarly, most of the isolated UPEC strains have strong cytotoxicity and can induce epithelial cells to form a strong host immune response, which is essential for defense against UTI. This result is consistent with previous study (Nam, 2013).

Conclusion

In conclusion, 7 canine UPEC strains were isolated from Yangzhou, the virulence factors and biofilm formation ability were identified. The results showed that the canine UPEC strains could invade T24 cells, induce cytotoxicity and activate the synthesis of proinflammatory cytokines in epithelial cells, indicating that canine UPEC has a certain potential of zoonosis.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20210621070617

Statement of conflict of interests

The authors have declared no conflict of interest.

Reference

Buberg, M.L., Mo, S.S., Sekse, C., Sunde, M., Wasteson, Y. and Witsø, I.L., 2021. *BMC Microbiol.*, **21**: 94. https://doi.org/10.1186/s12866-021-02160-y

Cai, W., Wannemuehler, Y. Dell'Anna, G., Nicholson, B., Barbieri, N.L., Kariyawasam, S., Feng, Y., Logue, C.M., Nolan, L.K. and Li, G., 2013. *PLoS Pathog.*, **9**: e1003428. https://doi.org/10.1371/journal.ppat.1003428

De Souza, G.M., Neto, E., da Silva, A.M., Iacia, M., Rodrigues, M., Cataneli Pereira, V. and Winkelstroter, L.K., 2019. *Infect. Drug Resist.*, **12**: 3595-3606. https://doi.org/10.2147/IDR.S228612

Dhakal, B.K., Kulesus, R.R. and Mulvey, M.A., 2008. *Eur. J. clin. Invest.*, **38**: 2-11. https://doi.org/10.1111/j.1365-2362.2008.01986.x

Duan, Q., Zhou, M., Zhu, X., Bao, W., Wu, S., Ruan, X., Zhang, W., Yang, Y., Zhu, J. and Zhu, G., 2012. *Vet. Microbiol.*, **160**: 132-140. https://doi.org/10.1016/j.vetmic.2012.05.015

Flores-Mireles, A.L., Walker, J.N., Caparon, M. and Hultgren, S.J., 2015. *Nat. Rev. Microbiol.*, **13**: 269-284.

Gibson, J.S., Cobbold, R.N., Kyaw-Tanner, M.T., Heisig, P. and Trott, D.J., 2010. *Vet. Microbiol.*, **146**: 161-166. https://doi.org/10.1016/j.vetmic.2010.04.012

Hu, Y., Dun, Y., Li, S., Zhao, S., Peng, N. and Liang, Y., 2014. *Asian Austral. J. Anim. Sci.*, **27**: 1131-1140. https://doi.org/10.5713/ajas.2013.13737

Johnson, J.R. and Stell, A.L., 2000. *J. Infect. Dis.*, **181**: 261-272. https://doi.org/10.1086/315217

Kathayat, D., Lokesh, D., Ranjit, S. and Rajashekara, G., 2021. *Pathogens*, **10**: 467. https://doi.org/10.3390/pathogens10040467

Leatham-Jensen, M.P., Mokszycki, M.E., Rowley, D.C., Deering, R., Camberg, J.L., Sokurenko, E.V., Tchesnokova, V.L., Frimodt-Møller, J., Krogfelt, K.A., Leth Nielsen, K., Frimodt-Møller, N., Sun, G., Cohen, P.S. and Blokesch, M., 2016. *mSphere*, **1**: e00055-15. https://doi.org/10.1128/mSphere.00055-15

Mabbett, A.N., Ulett, G.C., Watts, R.E., Tree, J.J., Totsika, M., Ong, C.Y., Wood, J.M., Monaghan, W., Looke, D.F., Nimmo, G.R., Svanborg, C. and Schembri, M.A., 2009. *Int. J. med. Microbiol.*, **299**: 53-63. https://doi.org/10.1016/j.ijmm.2008.06.003

Mitsumori, K., Terai, A., Yamamoto, S. and Yoshida, O., 1998. *FEMS Immunol. med. Microbiol.*, **21**: 261-268.

Mulvey, M.A., Lopez-Boado, Y.S., Wilson, C.L., Roth, R., Parks, W.C., Heuser, J. and Hultgren, S.J., 1998. *Science*, **282**: 1494-1497. https://doi.org/10.1126/science.282.5393.1494

Nagamatsu, K., Hannan, T.J., Guest, R.L., Kostakioti, M., Hadjifrangiskou, M., Binkley, J., Dodson, K., Raivio, T.L. and Hultgren, S.J., 2015. *Proc. natl. Acad. Sci. U.S.A.*, **112**: E871-E880. https://doi.org/10.1073/pnas.1500374112

Nakamura, Y., Yamamoto, N., Kino, Y., Yamamoto, N., Kamei, S., Mori, H., Kurokawa, K. and Nakashima, N., 2016. Establishment of a multi-species biofilm model and metatranscriptomic analysis of biofilm and planktonic cell communities. *Appl. Microbiol. Biotechnol.*, **100**: 7263-7279. https://doi.org/10.1007/s00253-016-7532-6

Nam, E., 2013. *J. Microbiol. Biotech.*, **23**: 422-429. https://doi.org/10.4014/jmb.1209.09051

Sharma, G., Sharma, S., Sharma, P., Chandola, D., Dang, S., Gupta, S. and Gabrani, R., 2016. *J. appl. Microbiol.*, **121**: 309-319. https://doi.org/10.1111/jam.13078

Shenagari, M., Bakhtiari, M., Mojtahedi, A. and Atrkar, R.Z., 2018. *Iran J. Basic med. Sci.*, **21**: 1226-1231.

Soto, S.M., Smithson, A., Martinez, J.A., Horcajada, J.P., Mensa, J. and Vila, J., 2007. *J. Urol.*, **177**: 365-368. https://doi.org/10.1016/j.juro.2006.08.081

Spaulding, C.N. and Hultgren, S.J., 2016. *Pathogens*, **5**: 30. https://doi.org/10.3390/pathogens5010030

Subashchandrabose, S., Hazen, T.H., Brumbaugh, A.R., Himpsl, S.D., Smith, S.N., Ernst, R.D., Rasko, D.A. and Mobley, H.L., 2014. *Proc. natl. Acad. Sci. U. S. A.*, **111**: 18327-18332. https://doi.org/10.1073/pnas.1415959112

Terlizzi, M.E., Gribaudo, G. and Maffei, M.E., 2017. *Front. Microbiol.*, **8**: 1566. https://doi.org/10.3389/fmicb.2017.01566

Wood, T.K., 2009. *Environ. Microbiol.*, **11**: 1-15. https://doi.org/10.1111/j.1462-2920.2008.01768.x

Xia, X., Meng, J., McDermott, P.F. and Zhao, S., 2011. *J. appl. Microbiol.*, **110**: 1166-1176. https://doi.org/10.1111/j.1365-2672.2011.04978.x

Yang, Y., Liu, Y., Zhou, M. and Zhu, G., 2018. *Pakistan J. Zool.*, **50**: 1807-1813. https://doi.org/10.17582/journal.pjz/2018.50.5.1807.1813