Epidemic Potential of *Escherichia coli* O16:H41-ST131: Compared with Pandemic O25b:H30-ST131 Lineage

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Background: O16:H41 is an important subclone among *Escherichia coli* (*E. coli*) sequence type (ST) 131, which has risen dramatically in recent years. However, reasons for the rapid increase of *E. coli* O16:H41-ST131 remain unclear. The aim of this study was to compare the pathogenicity and survivability features of *E. coli* O16:H41-ST131 with global epidemic O25b:H30-ST131 lineage.

Methods: Sixteen *E. coli* ST131 were divided into two groups: group O16:H41-ST131 (n=6) and group O25b:H30-ST131 (n=10). Adhesion and invasion activity of different isolates were measured using human T24 cells. Biofilm production was quantified by crystal violet staining. Fifty percent human serum was used to detect serum sensitivity. Resistance to hydrogen peroxide was detected by broth microdilution method, and anti-phagocytic function was determined by phagocytosis experiments.

Results: *E. coli* O16:H41-ST131 and O25b:H30-ST131 lineage showed similar biofilm formation, adhesion and invasion abilities. In terms of survivability, resistance to serum and hydrogen peroxide of *E. coli* O16:H41-ST131 was similar as that of *E. coli* O25b:H30-ST131. But anti-phagocytic function of *E. coli* O16:H41-ST131 was significantly weaker than that of *E. coli* O25b:H30-ST131.

Conclusion: The pathogenicity and survivability of *E. coli* O16:H41-ST131 were similar to those of *E. coli* O25b:H30-ST131, which may be important reasons for its increasing prevalence. Our study may contribute to a better understanding of the prevalence of *E. coli* O16:H41-ST131.

Keywords: O16:H41, O25b:H30, ST131, pathogenicity, survivability

Introduction

Escherichia coli (*E. coli*), which is commonly found in the gut flora of human beings and animals, can cause a wide range of infections such as septicemia, meningitis, and urinary tract infections.¹ Sequence type (ST) 131, identified in 2008, is currently recognized as a predominant lineage among *E. coli* worldwide.^{1,2} Almost all of *E. coli* ST131 isolates are resistant to fluoroquinolones and commonly carry extended-spectrum β -lactamase genes like *bla*_{CTX-M-15}.³ Therefore, the global increase in antibiotic resistance of *E. coli* is closely related to expansion of ST131 lineage.⁴ The epidemiological success of *E. coli* ST131 clonal group may be attributed to its enhanced pathogenicity and stronger survivability.⁵

O25b was generally considered as a predominant *E. coli* ST131 clonal serotype. However, the prevalence of *E. coli* O16-ST131 lineage significantly rose in recent

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years and made an important contribution to the expanding population of *E. coli*.^{4,6} A previous study showed that 33.7% of *E. coli* ST131 clinical isolates are O16 serotype in China.^{7,8} Besides, other research found that O16-ST131 lineage was a dominant lineage among fecal strains in China.⁸ In addition, the most prevalent lineage within *E. coli* ST131 is known as H30-ST131, which is often associated with many adverse consequences, such as multidrug-resistance (MDR) and persistent infections.⁹ Meanwhile, *E. coli* H41-ST131 is also a non-negligible lineage whose prevalence is even higher than that of *E. coli* H30-ST131 in certain regions.^{10,11}

E. coli ST131 can be classified into different clonal groups that differ in their virulence and survivability.⁴ Therefore, characteristics of different lineages should be described separately when exploring the causes of their epidemics. Our previous study showed that O25b-ST131 lineage (66.4%) was a dominant clinical *E. coli* ST131 lineage, followed by O16-ST131 subclone (33.6%).⁷ Our results suggested that O16-ST131 lineage was poised to become a major serotype of *E. coli* ST131 in China. Therefore, the aim of this study was to assess the difference in pathogenicity and survivability features between *E. coli* O16:H41-ST131 and global epidemic O25b:H30-ST131 lineage for a more comprehensive understanding of the epidemic potential of *E. coli* O16:H41-ST131.

Materials and Methods

Bacterial Isolates

16 *E. coli* ST131, collected from Union Hospital of Fujian Medical University and reported in our previous study,⁷ were randomly selected in this study. 16 *E. coli* ST131 were divided into two groups, group O16:H41-ST131 (n=6) and group O25b:H30-ST131 (n=10).

Pathogenicity Analysis

Adhesion Assay

Adhesion abilities were detected using human bladder cancer cells (T24 cells, Anchorage-dependent cell, FH0171, FuHeng Cell Center, Shanghai, China), performed as previously described.¹² Briefly, T24 cells were incubated with *E. coli* ST131 in 24-well plates at 37°C, 5% CO₂ for 3 hours and infected with multiplicity of infection (MOI) of 10. Thereafter, T24 cells were washed with 1×phosphate-buffered saline (PBS) thrice. Then T24 cells were lysed with 0.1% Triton X-100 for 10 minutes. The lysates were diluted serially and plated on MH-agar

plates for bacterial count. *E. coli* strain EC505 was used as positive control and *E. coli* DH5α served as negative control.

Invasion Assay

In invasion assay, the treatments of T24 cells were similar to those in adhesion assay but an additional step was carried out.¹² After 3 hours of incubation, medium was replaced with fresh medium containing 100μg/mL gentamicin and incubated for 1.5 hours to kill extracellular strains. Then T24 cells were washed with 1×PBS thrice and lysed with 0.1% Triton X-100 for 10 minutes. The lysates were diluted serially and plated on MH-agar plates for bacterial count.

Biofilm Formation Experiments

Biofilm formation capacity was quantified by crystal violet assay based on a method described in previous studies with a few modifications.^{13,14} The strains were grown overnight in LB broth medium at 37°C under stationary aerobic conditions. Then, 0.5 McFarland turbidity standard saline-washed cultures were diluted 1:100 in LB broth medium and 200 μL cell suspensions were inoculated into 96 flat-bottomed well, polystyrene microtiter plates. The cultures were incubated for 48 h at 37°C without shaking. After incubation, each well was washed with phosphate-buffered saline (PBS) to remove planktonic bacteria and fresh 200 μL LB broth medium with or without ciprofloxacin (CIP) (2μg/mL) was added to each well. Following incubation for 24 h, each well was gently washed with 200 μL of phosphate-buffered saline (PBS) three times and stained with 200 μL of 1% crystal violet for 15 min at room temperature. Then, the plates were washed with distilled water to remove excess dye. To quantify biofilm production, 200 μL of anhydrous ethanol was used to solubilize crystal violet. The optical density was measured at 595 nm in ELISA reader. LB broth medium without bacterial cultures was used as negative control (ODc) and *Enterococcus faecalis* (*E. faecalis*) ATCC 29212 served as positive control.¹⁵ The degree of biofilm production was classified according to the following criteria: strong (OD>2×ODc), moderate (1.5×ODc<OD≤2×ODc), weak (ODc<OD≤1.5×ODc), absent (OD≤ODc). The concentration of CIP was defined according to bioavailability in human urine.¹⁶

Viability Analysis

Serum Sensitivity Test

To analyze serum sensitivity, strains were incubated with 50% human serum as described previously.¹² Resistance to

serum was determined by the number of colonies (CFU/mL) recovered from each well.¹² *E. coli* strain EC505 was used as positive control and *E. coli* DH5 α served as negative control.

Hydrogen Peroxide Susceptibility Test

Hydrogen peroxide susceptibility was detected by broth micro-dilution method, according to the Clinical and Laboratory Standards Institute (CLSI, 2020) standards. Results were presented in the form of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). *E. coli* ATCC 25922 was used as quality control.

Phagocytosis Experiment

Anti-phagocytic functions were determined using RAW264.7 cells (Anchorage-dependent cell, FH0328, FuHeng Cell Center, Shanghai, China), performed as previously described.¹⁷ Briefly, 2×10^5 /mL RAW264.7 cells and 2×10^6 CFU/mL bacteria were incubated at 37°C for 1.5 hours. Trypan blue staining cell viability assay kit was used to determine viability of RAW264.7 cells. When phagocytosed, *E. coli* can be easily recognized under a microscope by Gram staining, which allows explicit and quantitative measurement of cell phagocytosis. Therefore, microscopy was used to visualize phagocytosed *E. coli*. Anti-phagocytic functions were observed in terms of anti-phagocytosis rate (PR): PR = (total numbers of cells harbored the phagocytosed *E. coli* in 200 cells)/200 $\times$ 100%. *E. coli* ATCC 25922 was used as positive control and *E. coli* DH5 α served as negative control. All experiments were conducted three times in three replicates.

Statistical Analysis

The Mann–Whitney *U* test was performed to assess differences in adhesion capabilities, invasion abilities, serum sensitivities, hydrogen peroxide susceptibilities, and biofilm formation capabilities between two groups. The two-sample *t*-test was conducted to evaluate the variation in anti-phagocytic function between two groups (*E. coli* O25b:H30-S131 and *E. coli* O16:H41-ST131) and the relationship between biofilm formation and antibiotics. All analyses were performed in SPSS-25 with a significance level of $\alpha = 0.05$.

Results

Pathogenic Potentials

Adhesion and Invasion Capabilities

Our study showed that adhesion and invasion abilities of two groups were significantly higher than those of *E. coli*

DH5 α (Figure 1, $p < 0.05$). But the results showed no significant difference in adhesion and invasion abilities between two groups (*E. coli* O25b:H30-S131 and *E. coli* O16:H41-ST131) (Figure 1, $p < 0.05$).

Biofilm Formation Capability

As quantified by crystal violet staining, all isolates were able to form biofilms in LB broth medium with or without CIP. Among them, 83.33% of *E. coli* O16:H41-ST131 and 70% of *E. coli* O25b:H30-ST131 showed weak biofilm forming ability. Although 83.33% of *E. coli* O16:H41-ST131 still presented weak biofilm forming ability, CIP significantly reduced biofilm biomass of 50% of *E. coli* O16:H41-ST131 (Table 1). In group O25b:H30-ST131, strong biofilm forming strains EC549 showed a significant reduction in biofilm forming ability ($t=19.067$, $p < 0.05$) and it showed weak biofilm forming ability in LB broth with CIP. There was no significant difference between two groups (*E. coli* O25b:H30-S131 and O16:H41-ST131 lineage) in LB broth medium with or without CIP observed ($p > 0.05$). The results of biofilm formation assay were shown in Table 1.

Survival Capacity

Serum Sensitivity

In our study, serum sensitivity was detected using 50% human serum. *E. coli* O16:H41-ST131 and *E. coli* O25b:H30-ST131 showed significantly higher resistance to serum than *E. coli* DH5 α (Figure 2A, $p < 0.05$). But two groups showed similar resistance to human serum ($p > 0.05$).

Susceptibility to Hydrogen Peroxide

In this study, the MIC value of hydrogen peroxide for all *E. coli* ST131 was 16 μ g/mL or higher. The MIC and MBC values of hydrogen peroxide for *E. coli* ATCC 25922 were all 8 μ g/mL (Table 2). It was observed that there were significant differences between *E. coli* O16:H41-ST131 and *E. coli* ATCC 25922 both in MIC and MBC. But no significant difference in resistance to hydrogen peroxide was observed between two groups of isolates (*E. coli* O16:H41-ST131 and *E. coli* O25b:H30-ST131 lineage) at MIC level (Figure 2B) and MBC level (Figure 2C).

Anti-Phagocytic Function

The results of anti-phagocytic function assay were shown in Figure 2D. The phagocytosis of *E. coli* O16:H41-ST131 by RAW264.7 cells was significantly lower than that of

Figure 1 Pathogenic potentials of *E. coli* O16:H41-ST131 and *E. coli* O25b:H30-ST131. (A) Adhesion ability on T24 cells. (B) Invasion ability on T24 cells.

Notes: * $p < 0.05$, ** $p < 0.01$.

Abbreviations: *E. coli*, *Escherichia coli*; ST, sequence type.

E. coli DH5α ($t=7.418$, $p < 0.001$). Meanwhile, anti-phagocytic function of *E. coli* O16:H41-ST131 was significantly weaker than that of *E. coli* O25b:H30-ST131 ($t=-2.527$, $p < 0.05$).

Discussion

Within the *E. coli* population, ST131 is a dominant MDR lineage worldwide.⁴ Recent studies showed that ST131 lineage carried more virulence genes, compared with other *E. coli* lineages.¹⁸ It was conventionally assumed that O25b was a predominant ST131 serotype, but the prevalence of *E. coli* O16-ST131 significantly rose in recent years.^{7,8} Different *E. coli* ST131 clone groups possess various virulence and survivability features.¹⁵ Therefore, in order to further explore the reasons for increased prevalence of *E. coli* O16:H41-ST131, we analyzed the difference in pathogenicity and survivability between *E. coli* O16:H41-ST131 and global epidemic O25b:H30-ST131 lineage.

In this study, adhesion, invasion and biofilm formation capabilities were used to assess pathogenicity. Previous studies suggested that adhesion and invasion capabilities seemed to be the key of bacterial pathogenesis and important factors of some adverse events such as antibiotic resistance and bacterial persistence.^{19,20} Our study showed that both groups of isolates (*E. coli* O16:H41-ST131 and *E. coli* O25b:H30-ST131) exhibited strong adhesion and

invasion ability to human bladder cancer cells, consistent with previous studies²¹ (Figure 1). At the same time, our study found that *E. coli* O16:H41-ST131 and pandemic *E. coli* O25b:H30-ST131 exhibited similar adhesion and invasion abilities (Figure 1, $p > 0.05$). This result suggested that *E. coli* O16:H41-ST131, like O25b:H30, might be able to colonize and establish infections when they enter human tissue. Occurrence of infections and enhanced antibiotic resistance are also closely related to the biofilm formation capacity of bacteria.²² It is hard for antibiotics to penetrate the biofilm, so the bacteria in biofilm are easy to become tolerant and resistant to antibiotics.²³ Meanwhile, it was found that weak biofilm forming strains induced weaker immune responses than strong biofilm forming strains, possibly leading to immune evasion.²⁴ In our study, we found that all of *E. coli* ST131 were able to form biofilms and the majority of *E. coli* O25b:H30-ST131 (70%) and *E. coli* O16:H41-ST131 (83.3%) presented ($p > 0.05$) weak biofilm formation ability, consistent with previous studies.²⁵ All strains used in our study were resistant to fluoroquinolones, so we also assessed their biofilm formation capacity post addition of CIP. Although CIP produced a significant reduction in biofilm biomass of 50% of *E. coli* O16:H41-ST131 and 30% of *E. coli* O25b:H30-ST131, all strains were still able to form biofilms.

Table I Biofilm Formation Capability Among Different *E. coli*-ST131 Isolates

Strains	Lineage	CIP ($\mu\text{g/mL}$)	Mean OD	Biofilm Formation Ability	t-value	p-value
EC662	O16:H41	0 3	0.124(± 0.004) 0.099(± 0.003)	Weak Weak	-5.572	0.005
EC620	O16:H41	0 3	0.144(± 0.007) 0.109(± 0.014)	Weak Weak	3.751	0.020
EC623	O16:H41	0 3	0.118(± 0.005) 0.1(± 0.004)	Weak Weak	7.76	0.010
EC578	O16:H41	0 3	0.098(± 0.014) 0.098(± 0.004)	Weak Weak	0.473	0.965
EC627	O16:41	0 3	0.103(± 0.011) 0.103(± 0.02)	Weak Weak	0.041	0.970
EC525	O16:H41	0 3	0.255(± 0.034) 0.237(± 0.027)	Strong Strong	0.714	0.515
EC549	O25b:H30	0 3	0.218(± 0.009) 0.1(± 0.007)	Strong Weak	19.067	$<10^{-3}$
EC541	O25b:H30	0 3	0.152(± 0.024) 0.099(± 0.005)	Moderate Weak	3.721	0.020
EC640	O25b:H30	0 3	0.151(± 0.014) 0.105(± 0.012)	Moderate Weak	4.197	0.014
EC586	O25b:H30	0 3	0.109(± 0.002) 0.099(± 0.009)	Weak Weak	1.489	0.233
EC668	O25b:H30	0 3	0.122(± 0.002) 0.12(± 0.003)	Weak Weak	0.765	0.487
EC587	O25b:H30	0 3	0.136(± 0.005) 0.132(± 0.011)	Weak Weak	0.538	0.628
EC674	O25b:H30	0 3	0.144(± 0.023) 0.13(± 0.185)	Weak Weak	0.813	0.462
EC570	O25b:H30	0 3	0.112(± 0.004) 0.103(± 0.011)	Weak Weak	1.08	0.545
EC545	O25b:H30	0 3	0.112(± 0.002) 0.106(± 0.008)	Weak Weak	1.257	0.328
EC568	O25b:H30	0 3	0.111(± 0.006) 0.111(± 0.003)	Weak Weak	0.088	0.934

Notes: Mean OD values $\pm$ standard deviation of each group of antibiotics; P value ≤ 0.05 was considered significant.

Abbreviation: CIP, Ciprofloxacin.

Our results suggested that refractory infections caused by *E. coli* O16:H41-ST131 might be related to its biofilm formation capacity.

Strong survivability is also a contributory factor to prevalence of pathogenic bacteria. In our study, serum resistance, hydrogen peroxide resistance, and anti-phagocytic function were used to assess survivability. Serum resistance and anti-phagocytic function were considered as important

determinants for survival of bacteria in vivo.^{12,26} Our study found that *E. coli* O16:H41-ST131 was capable of growing in human serum, indicating strong serum resistance capacity of this lineage. Serum resistance is one of the important mechanisms of bacteria enabling them to survive in the bloodstream of the host.¹² We supposed that the strong serum resistance might contribute to the current increase in bloodstream infections caused by *E. coli* O16:H41-ST131.

Figure 2 Survival capacity of *E. coli* O16:H41-ST131 and *E. coli* O25b:H30-ST131. **(A)** Resistance to serum bactericidal activity against 50% human serum. **(B)** MIC value of hydrogen peroxide. **(C)** MBC value of hydrogen peroxide. **(D)** Anti-phagocytic activity to RAW264.7 cells.

Notes: * $p < 0.05$, ** $p < 0.001$.

Abbreviations: *E. coli*, *Escherichia coli*; ST, sequence type; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

Besides, our study illustrated that anti-phagocytic function of *E. coli* O16:H41-ST131 was lower than that of *E. coli* O25b:H30-ST131, but still significantly higher than that of *E. coli* DH5α (Figure 2, $p < 0.05$). Anti-phagocytic function of bacteria plays an important role in the occurrence of infections. Strong anti-phagocytic function can protect *E. coli* strains from phagocytosis and ensure their survival in vivo, which conduces to causing infections. These results suggested that not only pandemic *E. coli* O25b:H30-ST131 had resistance to bactericidal effect of human immune system, but *E. coli* O16:H41-ST131 could resist human immune function and

cause infections. Resistance to hydrogen peroxide, often used as disinfectant in hospitals, seems to be closely related to hospital-related bacterial infections.²⁷ Two groups of strains (*E. coli* O16:H41-ST131 and *E. coli* O25b:H30-ST131) showed similar hydrogen peroxide resistance, which was significantly higher than that of *E. coli* ATCC 25922 (Figure 2, $p < 0.05$). As discussed previously, our results proved that *E. coli* O16:H41-ST131 and epidemic *E. coli* O25b:H30-ST131 showed similar pathogenicity and survivability features, which might contribute to the epidemiological success of *E. coli* O16:H41-ST131.

Table 2 Antibacterial Activity of Hydrogen Peroxide Against *E. coli* O16:H41-ST131 and *E. coli* O25b:H30-ST131

Strains	Hydrogen Peroxide	
	MIC(mg/mL)	MBC(mg/mL)
<i>E. coli</i> 662	16	16
<i>E. coli</i> 620	16	16
<i>E. coli</i> 623	32	32
<i>E. coli</i> 627	32	32
<i>E. coli</i> 578	16	32
<i>E. coli</i> 525	16	32
<i>E. coli</i> 640	16	32
<i>E. coli</i> 674	16	32
<i>E. coli</i> 668	32	32
<i>E. coli</i> 587	32	32
<i>E. coli</i> 570	32	32
<i>E. coli</i> 568	16	32
<i>E. coli</i> 586	16	16
<i>E. coli</i> 541	16	16
<i>E. coli</i> 545	32	16
<i>E. coli</i> 549	16	32
<i>E. coli</i> ATCC25922	8	8

However, there are some limitations to our study which should be considered in further research to better understand the reasons for increasing prevalence of *E. coli* O16:H41-ST131. First, the sample size of *E. coli* strains O16:H41-ST131 (n=6) and group O25b:H30-ST131 (n=10) in our study was small. All strains were collected from the same hospital so they might have a genetic relationship. Therefore, multi-center research with a large sample size should be conducted in our future studies for a more comprehensive understanding of survivability and pathogenicity of ST131. Second, our study only assessed the number of phagocytosed bacteria, but did not detect the survival of bacteria in RAW 264.7 cells. The survival of bacteria in RAW 264.7 cells might contribute to a better understanding of the pathogenesis of *E. coli* O16:H41-ST131 in vivo, so further research is still needed.

Conclusion

In this study, the pathogenicity and survivability features of *E. coli* O16:H41-ST131 were analyzed. The pathogenicity and survivability of *E. coli* O16:H41-ST131 were similar to those of pandemic O25b:H30-ST131 lineage. But in terms of anti-phagocytic function, *E. coli* O16:H41-ST131 was slightly inferior to O25b:H30-ST131 lineage. Our research could contribute to a comprehensive understanding of the

increasing prevalence of *E. coli* O16:H41-ST131 and suggests that it should be continuously monitored to cope with the increase in infections caused by *E. coli* ST131.

Abbreviations

ST, sequence type; CIP, ciprofloxacin; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; PR, anti-phagocytosis rate.

Ethics Approval and Informed Consent

All procedures of this study involving humans (individuals, medical records, human samples, clinical isolates and human cell lines) were reviewed and approved by the Medical Ethics Committee of Fujian Medical University Union Hospital (2020KY0121). All the patients participating in this study signed informed consent, while the guardians of children aged less than 18 years signed on behalf of them. We confirm that this study was conducted in accordance with the Declaration of Helsinki.

Consent for Publication

All authors confirm that the details of any images, videos, recordings, etc can be published.

Author Contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

Funding

This work was supported by the Joint Funds for the innovation of science and Technology, Fujian province [Grant number: 2017Y9049] and the Educational and Scientific Research Project for Young and Middle-Aged Teachers of Fujian Province (Grant number: JAT190191).

Disclosure

The authors report no conflicts of interest in this work.

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