Journal of Microbiology, Immunology and Infection (2015) 48, 329–334

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ORIGINAL ARTICLE

Phenotypes of *Escherichia coli* isolated from urine: Differences between extended-spectrum β-lactamase producers and sensitive strains

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Received 25 February 2014; received in revised form 12 April 2014; accepted 14 April 2014
Available online 24 May 2014

KEYWORDS
biochemistry;
Escherichia coli;
extended-spectrum β-lactamase;
phenotype

Background: *Escherichia coli* is a frequent causative agent of urinary tract infections, and increasing resistance of *E. coli* to antimicrobials presents a growing challenge.
Methods: Here we compare phenotypes of extended-spectrum β-lactamase (ESBL) producers ($n = 220$) with a control group of sensitive strains (non-ESBL producers; $n = 150$). For each strain, we assessed the presence of O25 antigen, hemolysis, biofilm production, sensitivity to antibiotics, and biochemical profile.
Results: Compared to the control group, ESBL producers were more frequently O25 positive (6.0% vs. 42.3%) and less frequently hemolytic (34.7% vs. 6.4%). Comparison of biofilm production in brain–heart infusion (BHI) and in BHI with 4% glucose supplementation showed that ESBL-positive strains produced biofilm in BHI with glucose less intensely than the control group ($p < 0.05$). Most ESBL producers were ciprofloxacin-resistant (91.8%). Biochemical analyses revealed that ESBL producers more frequently utilized inositol, ornithine, sorbitol, melibiose, and saccharose, whereas the control group more frequently used esculin, lysine, arginine, and dulcitol. The control group strains with O25 antigen were more commonly resistant to ciprofloxacin ($p < 0.05$). Pulsed-field gel electrophoresis results showed higher variability among the control group of sensitive strains.
Conclusion: These findings suggest a potential to detect ESBL strains based on virulence factors

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http://dx.doi.org/10.1016/j.jmii.2014.04.010

and biochemical properties, which could be useful in shaping proper empiric antimicrobial therapy, and for initiating such therapy as soon as possible.

Introduction

Escherichia coli is one of the most frequently isolated causative agents of urinary tract infections (UTIs), and is responsible for about 50% of nosocomial UTIs,[1,2] as well as 70–95% of community-acquired infections.[1,2] UTIs are usually mild infections; however, they have the potential to lead to sepsis and death.[3] Severe UTIs are especially dangerous to immunodeficient individuals, patients with congenital abnormalities,[4] obstruction of the urinary tract,[1] and diabetics.[5,6] Characteristic virulence factors in uropathogenic *E. coli* strains include cytotoxic necrotizing factors, α-hemolysin, adhesins, siderophores, and resistance to complement,[7] the presence of which influences *E. coli* survival in the urinary tract.

Molecular biology analyses indicate that the vast majority of extraintestinal *E. coli* belong to the phylogenetic group B2.[8,9] Although the strains of this group possess the highest number of specific genes, studies have found no specific virulence gene differentiating extraintestinal strains from gut commensal *E. coli*.[8,10] This suggests that the extraintestinal strains have developed from the gut ones.[10] Intestinal strains remain a frequent cause of UTIs in otherwise healthy individuals.[1] Transfer of *E. coli* from the gut to the urinary tract causes rapid changes in bacterial metabolism.[8] The gut environment is nutrient rich, with plenty of carbohydrates to support *E. coli* metabolism. By contrast, the urinary tract environment typically has a high osmolarity and abundance of nitrogenic compounds, but lacks oxygen and iron.[8] In response to the low concentration of iron in urine, bacteria express an array of siderophores.[11] *E. coli* uses short peptides and amino acids as a source of energy,[8] and the presence of accessible nutrients affects individual metabolic processes.[8,12]

For many years, the treatment of UTIs caused by *E. coli* was straightforward. However, this situation changed in the 1980s when resistance to high-generation cephalosporins emerged,[13] and was quickly transferred to *E. coli*. Treatment has been substantially complicated by the spreading of extended-spectrum β-lactamase (ESBL) producers along with other types of resistance (e.g., resistance to aminoglycosides and carbapenems).[3] Recent reports have described the growing importance of the O25b:H4-ST131 strain, which is linked to multiresistance,[14] especially to fluoroquinolones.[15,16] Strains bearing the O25 antigen frequently cause life-threatening complications in renal transplant patients.[17]

Additionally, catheterized patients are at high risk of biofilm development in the catheter. Biofilm production is affected by a range of external factors and is highly dependent on bacterial metabolism, especially acetate metabolism.[18] Acetate formation can be influenced by glucose concentration.[12] Growth in a biofilm, when compared with planktonic, substantially reduces bacterial antibiotic sensitivity and influences the growth rate. Growth of bacteria in a biofilm may also indicate higher clinical significance of an isolate.[19]

Early diagnosis of UTI causative agents, especially those that are antibiotic resistant, is crucial to adequate antibiotic therapy. The development of molecular biological methods has improved diagnostic accuracy; however, such methods are also more expensive and potentially more time consuming. Classical (nonmolecular) methods have the advantages of lower cost and the fact they can be processed at almost any laboratory without a need for the expensive facilities that are required for molecular biological methods. Studies have reported that antimicrobial resistance among uropathogenic *E. coli* is influenced by phenotypic features,[20,21] the loss of virulence factors,[21] or even bacterial metabolism.[8] Thus, the present study aimed to compare phenotypic features of ESBL-positive urinary isolates of *E. coli* with strains that are sensitive to basic β-lactam antibiotics (e.g., ampicillin and cephalothin), and to assess whether they could be distinguished based on the presence of certain virulence factors and their biochemical properties.

Materials and methods

Between 2008 and 2011, we collected 220 strains of ESBL-producing *E. coli* from urine as a part of the routine UTI diagnostics at the Department of Microbiology, St Anne's Teaching Hospital and the Faculty of Medicine, Masaryk University, Brno, Czech Republic. All strains were isolated in a quantity of at least 10^5 colony-forming units/mL. As controls, we used 150 strains of ESBL-negative *E. coli* sensitive to the following β-lactam antibiotics: ampicillin, amoxicillin-clavulanate, cephalothin, ceftazidime, and cefotaxime. The strains were divided according to their origin: hospital strains and strains from outpatient clinics.

Antibiotic sensitivity, hemolysis, and serotyping

In all strains, we evaluated sensitivity to ciprofloxacin, ertapenem, and gentamicin using the disc sensitivity test according to the Clinical and Laboratory Standards Institute criteria. For ESBL assessment, we used both the double-disc synergy test, and the Clinical and Laboratory Standards Institute test.[22] Only ESBL-positive/ampC-negative strains were included in the study.

Hemolysis was tested on blood agar with sheep erythrocytes. After overnight cultivation at 37°C, we assessed

hemolysis as a clear zone around individual colonies. To assess the number of strains with O25 antigen in each group, we used antiserum O25 (Denka Seiken, Tokyo, Japan) to perform the agglutination test. In positive strains, agglutination was visible within 1 minute.

Biofilms

Biofilm production was tested in microtitration plates for tissue cultures. Biofilm production was tested in brain–heart infusion (BHI), using the method of Stepanović et al.[23] The results were interpreted as described by Stepanović et al.[23] Similarly, we also tested biofilm production in BHI (HiMedia Laboratories, Mumbai, India) with 4% glucose supplementation.

Biochemical profiling

Biochemical profiles were assessed using ENTEROtest 24 (Erba Lachema, Brno, Czech Republic), which assesses utilization of indole, hydrogen sulfide, lysine, ornithine, urease, arginine, Simmons-citrate, malonate, phenylalanine, β-galactosidase, inositol, adonitol, cellobiose, saccharose, trehalose, mannitol, acetoin, esculin, sorbitol, rhamnose, melibiose, raffinose, dulcitol, and glucose. To each substrate, we added 100 μL of the strain suspended in saline (McFarland 1.0). We used paraffin oil to test indole, hydrogen sulfide, lysine, ornithine, urease, and arginine utilization under anaerobic conditions. The plate was cultured overnight at 37°C, and the results read and recorded the next day according to the manufacturer's instructions.

Pulsed-field gel electrophoresis

Genetic diversity of the strains was assessed using pulsed-field gel electrophoresis (PFGE) analysis of 10% of the *E. coli* strains, which were randomly selected using SPSS version 18 (IBM Corp., Armonk, NY, USA). These strains were cultured and processed according to the instructions for Gram-negative bacteria.[24] Restriction analysis was performed using XbaI (New England BioLabs, Ipswich, MA, USA). The results were evaluated using BioNumerics 6.5 (Applied Maths, Kortrijk, Belgium) with unweighted pair group method with arithmetic mean analysis and Pearson correlation coefficient (0.5%). A phylogenetic dendrogram was constructed based on PFGE fingerprint profiles, and the fingerprint profiles of the ESBL and non-ESBL strains compared.

Statistical analysis

Statistical analysis was performed using the SPSS program. Categorical data were evaluated using Fisher's exact test. For comparison of biofilm development (i.e., for numerical data), the Wilcoxon test or Mann–Whitney test was used. In all cases, $p < 0.05$ were considered to be statistically significant.

Results

ESBL producers

A majority of the ESBL-producing strains were isolated from women (62.7%) and from elderly patients (median age, 72 years; range, 16–96 years). Most samples originated from bed units of Brno hospitals (79.1%). Among ESBL producers, the vast majority of strains were sensitive to gentamicin (97.7%) and ertapenem (100.0%), whereas only 8.2% of the strains were sensitive to ciprofloxacin. The ciprofloxacin-sensitive strains significantly differed from the rest of the group in an array of features. Compared with the rest of the group, the ciprofloxacin-sensitive strains more frequently produced biofilm in BHI ($p = 0.015$), showed greater use of lysine ($p = 0.021$) and raffinose ($p = 0.009$), and showed less use of saccharose ($p = 0.001$) and ornithine ($p = 0.011$). Biochemical analyses also showed that the strains isolated from hospitalized patients utilized lysine much less than strains isolated from outpatients ($p = 0.025$).

The ESBL-producing strains were rarely hemolytic (6.4%). O25 antigen was detected in 42.5% of ESBL producers; these strains were typically nonhemolytic ($p = 0.047$) and showed greater usage of inositol ($p = 0.013$), ornithine ($p = 0.037$), and saccharose ($p = 0.044$). Among the ESBL producers, only nonhemolytic strains utilized inositol ($p = 0.045$). Table 1 summarizes the abilities of ESBL producers to utilize individual substrates. The most variable results were obtained for lysine and saccharose. Lysine positive strains tended to be inositol positive ($p = 0.003$), raffinose positive ($p = 0.012$), and saccharose negative ($p = 0.001$). Strains utilizing saccharose tended to be ornithine negative ($p = 0.037$) and raffinose negative ($p < 0.001$), and more frequently produced biofilm in BHI with glucose ($p = 0.007$).

Table 1 Comparison of biochemical profiles among extended-spectrum β-lactamase (ESBL)-producing strains and the control group[a]

Substrate	Positive reactions (%)		Fisher's exact test (p)
	ESBL producers ($n = 220$)	Control group ($n = 150$)	
Lysine	63.2	93.3	<0.001
Ornithine	88.2	78.7	0.028
Arginine	8.6	47.3	<0.001
Inositol	20.9	0.0	<0.001
Saccharose	89.6	40.0	<0.001
Esculin	1.4	7.3	0.004
Sorbitol	99.1	95.3	0.018
Dulcitol	4.6	12.7	0.006
Melibiose	95.9	91.3	0.049

[a] No statistically significant difference was demonstrated for adonitol, cellobiose, trehalose, mannitol, rhamnose, or raffinose ($p > 0.05$). All reactions were positive for indole, β-galactosidase, and glucose. No reactions were positive for hydrogen sulfide, urease, Simmons citrate, malonate, phenylalanine, or acetoin.

ESBL-positive strains frequently produced biofilm in BHI (72.7%), and in BHI with 4% glucose supplement (53.7%). The optical densities of biofilm produced in the two broths did not differ significantly ($p = 0.148$). The ESBL-positive biofilm-producing strains were, according to Stepanović et al,[23] typically weak producers in both media.

Control group

Like the ESBL-producing strains, control strains were also isolated from predominantly female patients (77.3%), but the control strains were isolated from younger patients than those of the ESBL group. Within the control group, the *E. coli* strains isolated from males were more often hemolytic ($p = 0.041$), arginine positive ($p = 0.031$), and esculin positive ($p = 0.017$) than strains from females. The majority of strains (71.3%) were cultured from the urine of outpatients, or from samples sent from general practitioners. Compared with the strains from hospitalized patients, the control strains from outpatients and general practitioners less frequently produced biofilm in BHI ($p = 0.037$).

The control strains were highly sensitive to basic antibiotics, as well as to the other tested antimicrobials. All strains were sensitive to ertapenem and gentamicin, and 96.7% of strains were sensitive to ciprofloxacin. The ciprofloxacin-resistant strains were often also O25 positive ($p = 0.029$). About one-third of the strains were hemolytic (34.7%), and these strains less frequently produced biofilm in BHI with glucose ($p = 0.0399$) and showed greater use of ornithine ($p = 0.005$) and arginine ($p = 0.002$). Agglutination with O25 antigen was positive in only 6.0% of strains, and these strains more frequently utilized saccharose ($p = 0.032$).

Over half of the control strains produced biofilm in BHI (66.0%) and in BHI with glucose (68.7%). Optical density analysis confirmed differing biofilm production between the two broths ($p = 0.037$). The control strains were, according to Stepanović et al,[23] typically weak biofilm producers in BHI, and moderate biofilm producers in BHI with glucose. Moreover, biofilm in BHI was less frequently produced by the saccharose positive ($p = 0.014$) and esculin positive control strains ($p = 0.008$).

Table 1 summarizes the overall biochemical profile of the control group. Individual biochemical features varied in regards to esculin, ornithine, lysine, arginine, adonitol, raffinose, and saccharose. Esculin utilizing control strains tended to be rhamnose negative ($p = 0.020$) and raffinose positive ($p = 0.007$). Almost four-fifths of control strains were ornithine positive, and these strains were significantly more frequently lysine positive ($p = 0.001$), saccharose positive ($p < 0.001$), dulcitol negative ($p = 0.029$), and adonitol negative ($p = 0.007$). We also observed that the saccharose positive strains tended to also be raffinose positive ($p = 0.036$).

Comparison of phenotypic features between the ESBL group and the control group

A slight predominance of ESBL producers (59.5%) was in tested *E. coli* strains. ESBL producers were mostly isolated from hospitalized patients (79.1%), whereas control strains were more frequent in outpatients (71.3%). O25-positive strains were more frequently found in the ESBL group ($p < 0.001$), whereas hemolysis was more common among control strains ($p < 0.001$; Table 2). Biofilm production in BHI without supplementation did not differ significantly between the two groups ($p = 0.507$). However, in BHI supplemented with 4% glucose, the control group showed significantly more intense biofilm production than the ESBL group ($p = 0.029$). The Enterotest 24 results confirmed significant between-group differences in substrate utilization (Table 1). ESBL producers utilized significantly more inositol, ornithine, melibiose, sorbitol, and saccharose, whereas the control group utilized significantly more esculin, lysine, arginine, and dulcitol.

Genetic diversity analysis revealed much greater diversity among the control strains than among ESBL producers (Fig. 1). The majority of ESBL strains (72.7%) belonged to one group with a 70% similarity level. This group was divided into two subgroups: one with a 75%, and the other with a 90% similarity level. However, the majority (66.7%) of the control strains did not form a group with other control strains at a 70% similarity level (Fig. 1).

Discussion

Here we found that the ESBL-producing *E. coli* in our study differed from the sensitive strains in an array of properties and clinical characteristics. The ESBL-positive strains were isolated mostly from hospitalized patients, whereas the sensitive strains were more frequently community acquired. Both types of strains were predominantly isolated from females, as they generally suffer from UTIs more often.[25] We also found that *E. coli* virulence factors (e.g., hemolysis) and biochemical properties differed between sexes, indicating higher virulence of strains isolated from UTIs in males.

Although α-hemolysin is an important virulence factor in *E. coli*,[11] we found hemolysis in only 6.4% of our ESBL strains.

Table 2 Typical diagnostic features of extended-spectrum β-lactamase (ESBL) producers and control (non-ESBL) strains

Diagnostic features	Likely classification
1. Nonhemolytic strain	ESBL producer
2. O25 positive (often resistant to fluoroquinolones)	
3. Inositol/saccharose/ornithine positive	
4. Biofilm production not influenced by 4% glucose	
1. Hemolytic strain	Non-ESBL
2. Non-O25 antigen (often sensitive to fluoroquinolones)	
3. Lysine/arginine/esculin/dulcitol positive	
4. Biofilm production more intense with 4% glucose	

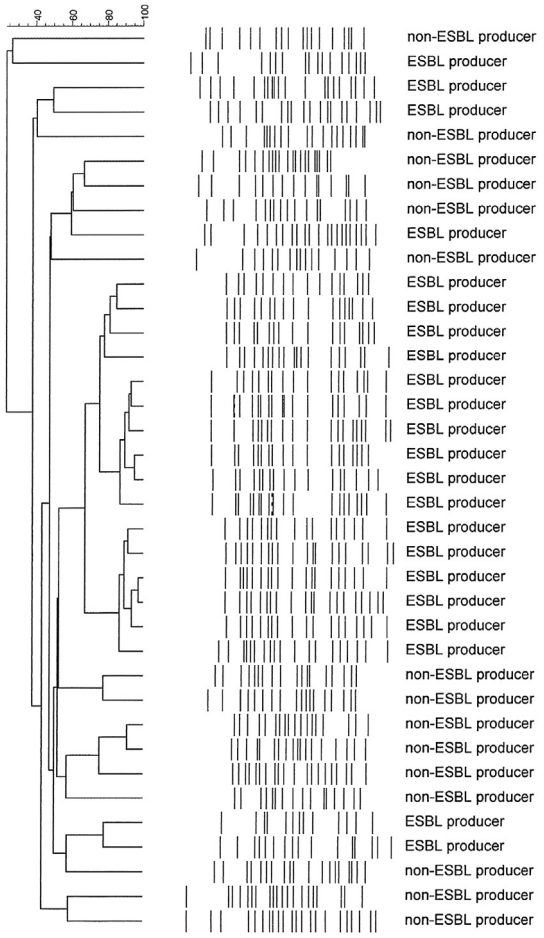

Figure 1. The phylogenetic dendrogram of the fingerprint profiles in the randomly selected group of extended-spectrum β-lactamase (ESBL) and non-ESBL producers.

By contrast, ESBL strains in India exhibit this feature much more frequently (65.6%).[26] Among our control strains, we found a higher percentage of hemolytic strains (34.7%), which reflects a previously reported 30—56% prevalence of the hly locus in uropathogenic *E. coli* strains.[11]

The *E. coli* strain O25b:H4-ST131 lacks some features that are typical for other *E. coli* strains, e.g., adhesins and α-hemolysin.[16] Thus, we assume that the low prevalence of hemolysis in our ESBL group might be caused by the relatively high number of O25-positive strains within this group (42.5% of ESBL producers). O25 strains also differed in some biochemical properties, for example, the O25 strains of both groups utilized saccharose more frequently than strains with other O antigens. Furthermore, we found that even O25-positive ESBL-negative strains were often resistant to ciprofloxacin (22.2%). This result is in agreement with studies demonstrating a correlation between CTX-M-15-O25b positivity and resistance to fluoroquinolones,[14] and highlights the importance of serotyping in the diagnosis of extraintestinal *E. coli* infections.

The β-lactamases of classes A and D are likely to be able to inhibit biofilm production.[20] We also noticed a positive link between biofilm production and saccharose utilization in ESBL producers. Compared with the sensitive strains, the ESBL strains less frequently produced biofilm in BHI with glucose, with the exception of saccharose-positive ESBL producers. Similarly, ciprofloxacin-sensitive ESBL strains more frequently produced biofilm in BHI without glucose, resembling non-ESBL strains in this regard. These findings indicate a possible relationship between biochemical properties of *E. coli* and pathogenicity expressed as biofilm production.

Compared with ESBL producers, the control strains exhibited higher variability of biochemical properties/utilized substrates, and this was supported by the PFGE results. However, there were also some important similarities between both *E. coli* groups, especially regarding saccharose utilization. Saccharose metabolism is considerably variable among pathogenic *E. coli* strains.[27] In addition to the link between the presence of the O25 antigen and saccharose-positivity, we found a link between ornithine-positivity and saccharose-positivity. By contrast, we noticed discordant results between saccharose and raffinose utilization. Within the ESBL group, these two substrates were utilized in indirect proportion to each other, whereas in the control group we noticed a direct proportion between the two. Differing saccharose and raffinose utilization by different clusters of *E. coli* has also been described.[28]

Interestingly, we also found a significant between-group difference in inositol utilization. No control strain was able to process inositol, whereas 20.9% of ESBL producers could. Moreover, inositol utilization was more common among O25-positive strains. It should be noted that inositol positivity is not a typical feature of *E. coli*, and a potential link to inositol phosphates has been previously discussed.[29] Inositol-positivity could be related to bacterial adhesion to the epithelial cells of the gut, in which a previous study demonstrated a short-term increase in inositol phosphate production, and the interference with signal mechanisms of the cells was assumed.[29] Alternatively, this feature might be related to inositol phosphate accumulation in the infected area, which could interfere with signal pathways and disrupt cell surfaces, potentially leading to cell death.[30]

External environment has a great influence on the resident bacteria, and there are known differences between the metabolism of gut and extraintestinal *E. coli*.[8] When bacteria are exposed to a new or hostile environment,[10] the bacterial cells that adapt best will survive. Bacteria living in the urinary tract can use various nutrients that are available due to the presence and continual production of urine.[31] The use of some substrates (e.g., lysine or arginine) could help *E. coli* to maintain homeostasis in the acidic environment typical for urine.[32]

In summary, the present comparison of *E. coli* phenotypes revealed numerous differences in biochemical properties between ESBL producers and non-ESBL strains, even though all strains were isolated from the same environment (urine). These findings suggest that ESBL strains can be detected and preliminary distinguished from sensitive strains on the ground of virulence factors and biochemical properties. This could be useful in shaping proper empiric antimicrobial therapy, and for initiating such therapy as soon as possible.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

This work was supported by a grant from the Ministry of Health of the Czech Republic (NS9665).

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