Adhesion and invasion to epithelial cells and motility of extended-spectrum β-lactamase-producing *Escherichia coli* reveal ST131 superiority: a comparative *in vitro* study of extraintestinal pathogenic *E. coli* lineages

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**Abstract**

**Purpose.** Extended-spectrum β-lactamase (ESBL)-producing extraintestinal pathogenic *Escherichia coli* (ExPEC) sequence type ST131 is pandemic, and it is the major contributor to antibiotic resistance in *E. coli*. Despite its epidemiological superiority, the physiological reasons that decipher its success remain elusive. We aimed to compare the adhesion, invasion and motility potential of ST131 versus other *E. coli* lineages.

**Methodology.** In this *in vitro* comparative study, 14 ESBL-producing ExPEC community-onset bacteremia isolates were chosen from a reported clinical collection (Karfunkel D, Carmeli Y, Chmelnitsky I, Kotlovsky T, Navon-Venezia S. *Eur J Clin Microbiol Infect Dis* 2013;32:513–521). Isolates were divided into two groups, ST131 (*n*=7) and ‘non-ST131’, sporadic sequence types (*n*=7). Virulence and adhesion genes were screened by PCR in all isolates. Virotyping and serotyping were performed for ST131 isolates. Adhesion and invasion were screened to Caco-2 epithelial cells, and motility on semi-solid agar were quantified and compared between the two groups. Fluorescence microscopy using anti-LPS *E. coli* antibodies was used for visualization and confirmation of adhesion and invasion.

**Results.** ST131 isolates belonged to the O25b:H4-B2 subclone. Two ST131 virotypes were found, A (two *bla*CTX-M-15 H30-Rx) and C (two *bla*CTX-M-15 H30-Rx and three *bla*CTX-M-14 H30 isolates). The average number of adhesion and virulence genes carried by ExPEC ST131 isolates and non-ST131 isolates was 5.3 and 3.7, respectively (*P*<0.05). Group analysis showed that ST131 surpassed non-ST131 lineages in all three physiological properties: adherence (17.1 vs 13.1 %, *P*<0.001), invasion (0.4 vs 0.17 %, *P*<0.01), and swarming motility on all media tested (*P*<0.05).

**Conclusion.** This study demonstrates ST131 superiority that may explain its improved gut-colonization and dissemination capabilities within the host. These insights are an important step in our understanding of ST131 epidemiological success.

**INTRODUCTION**

Extraintestinal pathogenic *Escherichia coli* (ExPEC) may cause a variety of infections including urinary tract infections and bacteremia [1, 2]. Among different ExPEC genetic lineages sequence type 131 (ST131) is currently recognized as pandemic, with occurrences worldwide [3]. Since its emergence, this clone has disseminated globally becoming the most widespread ESBL-producing genetic lineage, displacing other ExPEC strains in many countries [2]. ST131 lineage is known to cause both community-onset [4] and healthcare-associated infections [5]. It can colonize healthy humans, young and elderly, asymptotically [6, 7].

The mechanisms underlying its pandemic nature are yet to be defined. Previous studies showed that the epidemiological success of this clone compared to other non-ST131 *E. coli* lineages may be related to its multidrug resistance (MDR) phenotype and high virulence gene content [1]. An elevated metabolic potential demonstrated by a high assimilation of nutrients *in vitro* was also described as a possible contributor to its success [8]. ST131 isolates belong mainly
to phylogenetic group B2, known to be correlated with high extraintestinal virulence, and was also recognized to be associated with enhanced killing in a murine infection model [9]. Other studies indicated that although multidrug resistant and highly virulent, the severity of sepsis, bacteremia and mortality rates among patients affected by ST131 and non-ST131 isolates is similar [5, 10].

The epidemiological success of E. coli ST131 lineage was noticed also in Israel. In a previous study performed in a large tertiary medical centre in the Tel Aviv area, the incidence rate of community-onset bacteremia caused by ESBL-producing E. coli increased 2.5-fold during a 7-year period, due to the clonal expansion of the ST131 clone and its replacement over other ESBL-producing E. coli STs [4].

Here we demonstrate several physiological differences providing new support for the phenotypic advantages of this clone. We compared seven ST131 isolates and seven non-ST131 sporadic clones characterized previously [4], all isolates were ESBL-producing and caused community-onset bloodstream infections. The phenotypic studies included adhesion and invasion to Caco-2 cells, and swarming motility. We hypothesized that ST131 isolates possess enhanced adhesion, invasion and motility compared to other ExPEC lineages. These superior physiological properties may lead to an improved gut colonization and an efficient dissemination of ST131 within the host.

METHODS

Study design, bacterial strains and growth conditions

The study was designed as a comparative in vitro study consisting of 14 MDR ESBL-producing clinical ExPEC isolates. All isolates caused community-onset bloodstream infections recovered from individual patients [4]. Isolates were genotyped previously using SpeI restriction and PFGE, following analysis and comparison of the PFGE profiles according to the Dice similarity index (1.0 % tolerance interval) using the GelCompar II software, version 2.5 (Applied Maths, Kortrijk, Belgium; S. Navon-Venezia, unpublished data, supplementary material, Fig. S1, available in the online Supplementary Material). STs were assigned by MLST (http://bigsdb.pasteur.fr/ecoli/ecoli.html) [4].

The study isolates were divided into two groups based on their STs, PFGE and epidemiological occurrences: (i) ST131 group (n=7); (ii) non-ST131 isolates (n=7) (Figs 1 and S1) [4]. Bacterial strains were grown and maintained on Luria–Bertani (LB) agar plates or LB medium (HyLabs, Rehovot, Israel), supplemented with ampicillin (100 µg ml⁻¹) for maintenance of the ESBL-encoding plasmids.

Molecular characterization and virulence gene screening

The 14 isolates were classified into E. coli phylogroups using an improved multiplex PCR method [11]. The presence of CTX-M genes was determined by Karfunkel et al. [4] using Woodford’s multiplex PCR assays [12], followed by cloning and sequencing. PCR identification of ST131:O25b serotype was conducted based on the detection of SNPs in pabB and trpa genes [13]. Serotypes of H-antigens of ST131 isolates were identified by sequencing of internal parts of fliC gene obtained by PCR with flcC-1 and flcC-2 primers [14]. Sequencing was performed by HyLabs (Rehovot, Israel) and the results were analysed using the BioEdit software v7.2.5 [15]. H4-serotypes were compared with the published flcC-H4 sequence (AB028472) and verified by PCR of the entire flcC-H4 region [14]. Virotyping of ST131 isolates was performed using PCR [16]. Subclones of ST131 were identified based on the presence of fimH30 allele [17] and specific for H30-Rx subclone SNP in the ybbW, allantoin-encoding gene [18]. Presence of adhesion genes was assessed by PCRs described elsewhere: fimH, papC, sfaS, focG, gafD, bmaE, afa/draBC, rfaE [19], csgA [20], ecpA [21], sfa/focCD, iha and hra [22].

Adhesion and invasion assays

The interaction of E. coli ST131 and non-ST131 with the human Caco-2 epithelial cell line (ATCC HTB-37) was studied as previously described [23] with some modifications. Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 20 % (v/v) FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, penicillin (50 U ml⁻¹) and streptomycin (50 µg ml⁻¹) at 37 °C, 5 % CO₂. Monolayers of Caco-2 cells (2×10⁵ cells per well) were seeded in a 24-well plate and after 24 h were infected with bacteria at a m.o.i. of 10, centrifuged at 600 g for 5 min and then incubated at 37 °C, 5 % CO₂ for 30 min. The infecting dose of bacteria was confirmed by serial dilutions and plating on LB agar plates. Non-adherent bacteria were removed by washing with PBS three times. Caco-2 cell lysates were prepared by adding 1 ml of 0.1 % (v/v) Triton X-100 in PBS for 4 min at room temperature, and adherent bacteria were enumerated by serial dilutions and plating on LB agar plates. The percentage of adhesion was the ratio between the number of adhering bacteria and the initial infecting bacterial concentration. Enumeration of bacterial invasion to Caco-2 cells was assessed similarly, but with an incubation time of 1.5 h (37 °C, 5 % CO₂) followed with elimination of extracellular bacteria by gentamicin treatment (300 µg ml⁻¹) for 1 h. The working concentration of gentamicin used in the invasion assays was calibrated to fit the susceptibility of our study isolates. Removal of extracellular bacteria was validated by plating PBS used for cell wash after gentamicin treatment. The percentage of invasion was calculated by dividing the number of invading bacteria, resulted from plating serial dilutions of cell lysates following gentamicin treatment, by the initial bacterial concentration infecting the Caco-2 cells. Adhesion and invasion assays were performed in triplicate wells in three independent experiments. E. coli ST131 strain 76 was included in all adhesion and invasion assays as an internal reference strain and E. coli ATCC 25922 was included as a non-invasive negative control strain.
**Fluorescence microscopy for visualization of adhesion and invasion**

Adhesion and invasion of two representative ExPEC isolates, ST131 isolate 76 and non-ST131 isolate 63, were visualized using fluorescence microscopy. Adhesion and invasion experiments were conducted in six-well plates following the steps described in the previous section. Caco-2 cells with adhered or invaded bacteria were fixed with 3% paraformaldehyde for 20 min at room temperature, washed with PBS, and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min at room temperature. To prevent autofluorescence, samples were treated twice with NaBH₄ (1 mM in PBS) for 10 min. Following blocking with 1% BSA in PBS for 1 h at room temperature, E. coli LPS was detected with rabbit E. coli O antisera generated against multiple strains of E. coli LPS (Statens Serum Institute, Denmark), actin fibers were detected with Texas Red-Phalloidin (T7471, ThermoFisher Scientific, USA) and DNA was stained with Hoechst 33342. Images were acquired using Photometrics CoolSNAP HQ2 camera mounted on Olympus iX81 microscope and processed using ImageJ.

**Motility assays on semi-solid agar**

Motility assays were performed on semi-solid agar plates as previously described [24] using three different media including: LB, minimal medium M9 (containing Na₂HPO₄ 0.76%, KH₂PO₄ 0.3%, NaCl 0.05%, NH₄Cl 0.1%, glucose 0.4%, MgSO₄ 0.2M, CaCl₂ 0.01M) and urine (normal-pooled human urine). Semi-solid agar plates of each media tested were supplemented with 0.3% agar. Logarithmic bacterial cultures grown in aerated conditions (180 r.p.m.) were spotted (3 µl) in the centre of each plate and were incubated overnight at 37°C (16 h for LB agar plates and 20 h for M9 and urine agar plates). Growth zones after incubation were measured for motility evaluations. All semi-solid agar plates were freshly prepared and assays were performed in duplicates in two independent experiments. Non-motile Klebsiella pneumoniae ATCC 13883 was used as a negative control.

**Statistical analysis**

Results of adhesion, invasion and motility are expressed as the mean value±standard deviation (sd). The differences between ST131 and non-ST131 groups were assessed using nonparametric two-tailed Mann–Whitney test. Differences
in adhesion levels of bacteria possessing certain adhesion genes within each group, and effect of media on motility were analysed using two-tailed Mann–Whitney test. Associations between physiological trait values as well as the number of adhesion genes were tested for each group using nonparametric two-tailed Spearman correlation. Box and whisker graphs and statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, CA, USA). Differences among groups were considered statistically significant if \( P<0.05 \).

**RESULTS**

**Genetic characterization of ExPEC isolate and virulence gene content**

All 14 study isolates (seven pandemic ST131 and seven sporadic non-ST131 clones) were characterized for their phylogenetic groups, subclones, \( \text{bla}^{\text{CTX-M}} \), alleles, and for multiple virulence factors (Fig. 1). All ExPEC ST131 isolates belonged to phylogroup B2, whereas the sporadic non-ST131 isolates belonged to various phylogroups, including A, B2, D, E and F (Fig. 1). The majority of \( E.\ coli \) ST131 isolates belonged to virotype C and the remaining two, to virotype A. All ST131 isolates exhibited O25b and H4 antigens and possessed \( \text{fimH30} \) allele, and four isolates which carried the \( \text{bla}^{\text{CTX-M-15}} \) gene belonged to the H30-Rx subclone (Fig. 1). Among \( \text{bla}^{\text{CTX-M-15}} \)-type ESBLs, \( \text{bla}^{\text{CTX-M-15}} \) was the most dominant allele carried by four ST131 and three non-ST131 isolates. Positive PCR results of the tested virulence and adhesion genes revealed that the ExPEC ST131 group harboured a higher average number of adhesion and virulence genes compared to the non-ST131 group (5.3 vs 3.7, \( P<0.05 \), Fig. 1).

**Adhesion and invasion abilities of ExPEC isolates to human epithelial Caco-2 cells**

The adhesion and invasion abilities of ExPEC belonging to the pandemic ST131 clone were compared with sporadic ExPEC clones. ST131 isolates exhibited a statistically higher adhesion ability (\%) compared to non-ST131 strains (17.1±5 compared to 13.1±4.4, \( P<0.001 \), Fig. 2a). It should be noticed that all 14 ExPEC isolates adhered to Caco-2 cells, but their average adhesion level (%) varied significantly within groups: 11.3±4.3 to 25.4±2.8 for \( E.\ coli \) ST131, and 8.9±2.7 to 16.3±7 for non-ST131 isolates (average adhesion levels with SDs for each isolate are shown in Table S1).

The invasion to Caco-2 cells was assessed for nine isolates (five \( E.\ coli \) ST131 isolates and four \( E.\ coli \) non-ST131 strains). The average invasiveness of \( E.\ coli \) ST131 isolates was significantly higher than that of non-ST131 isolates (0.4±0.26 compared to 0.17±0.17, \( P<0.01 \), Fig. 2b). All tested isolates were found to invade Caco-2 cells, with high differences in their invasive potential and a wide range of variability within the two groups: 0.09±0.01 to 0.69±0.1 for the ST131 group and 0.06±0.02 to 0.42±0.11 for non-ST131 (Table S1).

We did not find a correlation between adhesion and invasion levels of isolates within each group (\( P>0.05 \)). Moreover, neither adhesion nor invasion correlated with the total number of adhesion genes (\( P>0.05 \)). In addition, when we examined the contribution of adhesion genes present in only several isolates to their adhesion level compared to isolates that lacked them within the same group (\( \text{afa/draBC} \) in ST131 lineage and \( \text{papC} \) and \( \text{iha} \) in non-ST131 isolates), we did not observe significant differences (\( P>0.05 \)).

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**Fig. 2.** Adhesion and invasion of ST131 and non-ST131 ExPEC to Caco-2 cells. The adhesion and invasion abilities of \( E.\ coli \) ST131 and non-ST131 into Caco-2 cells were studied by infecting cell monolayers (m.o.i. of 10). Adhesion (a) and invasion (b) levels were assessed by performing viable counts on LB agar plates. Percentage of adhesion or invasion was determined by the ratio of adhering or intracellular bacteria (c.f.u. ml\(^{-1}\)) to the initial bacterial concentration (c.f.u. ml\(^{-1}\)). Data presented in box and whisker plots (min to max) describe the mean adhesion or invasion level of each group, based on triplicates obtained from three independent experiments performed on each isolate tested. A, each box represents adhesion levels of seven ExPEC isolates. B, boxes describe the invasion level of five ST131 and four non-ST131 lineages. *** \( P\text{-value}<0.001 \); ** \( P\text{-value}<0.01 \).
Visualization of adhesion and invasion to Caco-2 cells using fluorescence microscopy

Adhesion and invasion of two ExPEC isolates: *E. coli* 76 (ST131 lineage) and *E. coli* 63 (non-ST131), were demonstrated using fluorescence microscopy (Fig. 3). Images show that both ExPEC isolates adhered and invaded to Caco-2 cells. *E. coli* ST131 76 displayed an enhanced adhesion and invasion ability, reflected by an increased number of bacteria per cell, corresponding to the adhesion and invasion levels (%) of both isolates, *E. coli* ST131 76 – 17.4±1.4 and 0.56 ±0.15, and *E. coli* non-ST131 63 – 13.3±2.2 and 0.06±0.02, respectively (Table S1).

Motility of ExPEC ST131 isolates vs non-ST131 isolates

In order to compare the swarming motility of ExPEC ST131 isolates with non-ST131 isolates, motility assays were performed. ST131 isolates exhibited enhanced swarming motility compared to non-ST131 isolates in all three media tested: LB, minimal and urine agar (Fig. 4). The average motility (cm) of *E. coli* ST131 compared to non-ST131 on rich LB agar was 3.3±2.0 to 1.6±1.2, respectively (*P*<0.05, Fig. 4a). On minimal agar media, although motility of all ExPEC tested was significantly lower compared to LB media (*P*<0.0001), ST131 isolates showed a higher average motility (cm) compared to non-ST131 isolates, 0.69±0.25 vs 0.46 ±0.26 on M9 agar (*P*<0.01), and 0.66±0.15 vs 0.44±0.08 on urine agar (*P*<0.0001, Fig. 4b). The motility data (average diameters with S DS) exhibited by each isolate are presented in Table S1. Motility did not correlate with adhesion or invasion levels and was independent with the number of adhesion genes (*P*>0.05). The only apparent correlation found for ST131 isolates was between motility on urine agar and their invasive rate (*P*<0.05).

DISCUSSION

In this comparative *in vitro* study, seven isolates belonging to the worldwide pandemic ExPEC ST131 clone were compared with seven non-ST131 ExPEC isolates belonging to other clonal lineages in order to define the physiological advantages of ST131 lineage. All 14 ExPEC were community-onset bacteremia isolates recovered from individual patients admitted to the same hospital [4]. All ST131 isolates were characterized as members of the O25b:H4-B2-ST131 clonal group, which was described in many countries.

![Fig. 3. Fluorescence microscopy of adhesion and invasion of *E. coli* ST131 isolate 76 and *E. coli* non-ST131 isolate 63. Caco-2 cells with adhering (a) and invading (b) bacteria were fixated with 3 % paraformaldehyde. Samples were immunostained for bacterial LPS (green). Cellular actin fibers and DNA were stained with Phalloidin (red) and Hoechst 33342 (cyan), respectively. Caco-2 cells without bacteria were used for control. The regions surrounded by rectangles in the merged panels were magnified in the zoom panels; arrows point to bacteria. Scale bar: 30 µm.](image-url)
The isolates were classified as virotypes A and C, described previously for ExPEC strains causing nosocomial bloodstream infections [16], and belonged to the fimH30 subclone. ST131 isolates carrying the \(\text{bla}_{\text{CTX-M-15}}\) gene were classified as a H30-Rx subclone. Five out of seven of the non-ST131 isolates (ST472, 473, 474, 475 and 477) were actually new STs identified for the first time [4], which allowed comparison of epidemic ST131 isolates and non-ST131 sporadic lineages.

We focused on three host–pathogen interactions: adhesion and invasion to epithelial cells that are the first steps during infection [27], and bacterial motility required for further dissemination inside the host [28]. These functions are performed by fimbriae and flagella that participate in different interactions within the host [29, 30]. Adhesion, invasion and motility are closely connected and seemed to be necessary for bacterial persistence and pathogenesis.

The data presented herein compared 14 ExPEC isolates (seven for each group) and revealed statistically significant differences \((P<0.05)\) between these two groups. ST131 group isolates surpassed non-ST131 group isolates in all three physiological properties evaluated; they showed significantly higher average levels of adhesion and invasion to Caco-2 cells, and pronounced motility. It should be noted that wide-range capability levels were identified for each ExPEC isolate; this reflects the importance of using large sets of isolates for comparative studies.

All ExPEC strains showed a certain level of adhesion to Caco-2 cells, but ST131 isolates exhibited 1.3-fold higher adhesion ability compared to non-ST131 strains \((17.1\pm 5\% \text{ compared to } 13.1\pm 4.4\%, P<0.001, \text{Fig. 2a})\). These findings may indicate an improved adhesion of this lineage to gut epithelial cells that may lead to a higher colonization potential and prolonged gastrointestinal existence, which may be important for extraintestinal dissemination. Peirano \textit{et al.} compared two isolates of ST131 with the other two internationally successful ExPEC strains – ST405 and ST101 (two isolates from each ST) – and found that ST131 showed the lowest adhesion level to HEp-2 and Caco-2 cells in the presence of mannose [31]. Mannose inhibits type-1 fimbriae-mediated adhesion and this might explain the results obtained [2]. Another possible explanation is that ST131 fails to show the adhesion advantage compared to other pandemic strains, but is indeed superior when compared to sporadic clones. Vimont \textit{et al.} demonstrated \textit{in vivo} that a CTX-M-15-producing ST131 strain, TN03, had the highest intestine colonization ability in mice relatively to the other three commensal \textit{E. coli} strains [26].

Together with enhanced adhesion, ExPEC ST131 surpassed sporadic ExPEC strains in its invasion capabilities too. The average invasion level of ST131 to Caco-2 cells was 2.5-fold higher than non-ST131 isolates \((0.4\pm 0.26\% \text{ compared to } 0.17\pm 0.17\%, P<0.01, \text{Fig. 2b})\). The average level of invasion exhibited by ST131 isolates to Caco-2 cells may indicate their enhanced ability to invade epithelial cells in general. We did not find a direct correlation between adhesion and
invasion levels (P>0.05), and indeed adhesion and invasion may be unrelated, as these two processes may involve different molecular mechanisms [23].

The motility is highly important for ExPEC pathogenicity. It was described previously as one of the characteristics that distinguishes between pathogenic and commensal ExPEC strains in humans [29], and may likely contribute to the early colonization of the urinary tract [24]. All tested ExPEC isolates revealed some motile potential: pronounced on rich LB agar and decreased dramatically on two minimal media, M9 agar and urine agar. ST131 isolates were significantly more motile compared to non-ST131 isolates on all media tested (P<0.05). An ability of the ST131 clone to be more motile on urine-containing agar also might be associated with enhanced dispersion inside the body and may suggest an explanation for the high prevalence of this clone among urinary tract infections [7, 26].

E. coli isolates were tested for the presence of fimbria- and flagella-encoding genes and also non-fimbrial adhesins. The presence of fimH in all isolates may explain, in part, their invasive abilities, as strains lacking this gene failed to invade [23]. The iha gene was found in all ST131 isolates, and was previously recognized (with the fimH gene) as characteristic for that clone [3]. A mutation in the iha gene decreased colonization in ligated pig intestine [32]. The total number of adhesion genes carried by each isolate did not seem to correlate with adhesion, invasion or swarming motility levels. This can be explained by the possible involvement of additional adhesins or by varying expression levels that may contribute to the phenotypes observed. Specifically for the ST131 clone, it was shown that higher expression levels of flagella genes correlated with enhanced adhesion and invasion to T24 bladder epithelial cells [30].

In conclusion, ST131 ExPEC isolates possess a significant superiority in adhesion and invasion to Caco-2 epithelial cells, and show pronounced motility compared to other ExPEC genetic lineages. These findings may propose possible advantages to ST131 in gut epithelial cell colonization, together with superior extraintestinal dissemination potential. These functional findings should be further assessed in vivo as they may contribute to our understanding of the high worldwide occurrence and dissemination of this pandemic clone.

References


