Phylogenetic grouping and virulence characterization of ESBL-producing and non-producing vaginal *Escherichia coli*

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Abstract: The initial colonization of the vaginal mucosa with *Escherichia coli* is considered as a critical step toward urinary tract and neonatal infections. This study was conducted to characterize ESBL-producing (n=40) vaginal *E. coli* isolates from pregnant and non-pregnant women. These isolates were compared with corresponding ESBL-non-producing *E. coli* isolates (n=21). Both groups were investigated using PCR-based protocols for their phylogenetic origin and virulence genotype. High numbers of ESBL producers and non-producers were from group B2 (47.5% vs. 42.8%, respectively). None of ESBL non-producers clustered in group D, whereas significant numbers ($P \leq 0.05$) of them belonged to group B1 (33.3%) in comparison with 20.0% and 7.5% of ESBL producers, respectively. Significant differences in the prevalence of this study included virulence factors were not observed between these two groups. In both high rates of multiple virulence factors possession were demonstrated among isolates belonged to groups B2 and D. Comparison of CTX-M-producers with non-CTX-M-ESBL-producers and ESBL-non-producers revealed no significant differences among these three groups. In total, 60.8%, 17.3%, 17.3% and 4.3% of multidrug resistant (MDR) isolates clustered in groups B2, D, A and B1, respectively. All MDR-ESBL-non producers (100%) belonged to phylogroup B2 compared with 50% of MDR ESBL-producers and their virulence was much more higher. This study indicates that significant differences are not present between ESBL-producing and non-producing vaginal *E. coli* for both phylogenetic group distribution and virulence genes possession. Also, ESBL-producing vaginal *E. coli*, especially CTX-M producers, tend to be more dominant among the highly virulent phylogroup B2 and to a lesser extent group D. These data reveal the importance of vaginal colonization by these highly virulent, MDR, ESBL-producing *E. coli* as a source of extraintestinal *E. coli* infections.

Keywords: Vaginal *E. Coli*, ESBL Producers, Virulence, Phylogroups

1. Introduction

Vaginal colonization by *Escherichia coli* was documented as a source of other extraintestinal *E. coli* infections in both women and their contacts, such as urinary tract infections (UTIs) [1] and neonatal infections [2]. In addition, sexual transmission of *E. coli* between partners was reported [3]. Vaginal *E. coli* was also reported as one of the predominant microorganisms in cases of aerobic vaginitis [4-6]. In theory, aerobic vaginitis may be a better candidate than bacterial vaginosis as the cause of pregnancy complications, such as ascending chorioamnionitis, preterm rupture of the membranes and preterm delivery [4]. An extensive analyses of phylogenetic group and virulence factors of *E. coli*

isolated from females reproductive tract infection (RTI) were carried out [7-10]. In these studies, it was found that vaginal *E. coli* have unique properties that may enhance their virulence. These properties are similar to those associated with other extraintestinal pathogenic *E. coli*, where most of them were derived from phylogroups B2 and D and possess numerous virulence factors such as adhesins, toxins, siderophores and polysaccharide coatings.

Extended-spectrum beta-lactamases (ESBLs) are a group of enzymes mediating resistance to most beta-lactams, including expanded-spectrum cephalosporins but excluding carbapenems and cephamycins. These enzymes are now widely distributed worldwide in Gram-negative bacteria (particularly in Enterobacteriaceae) with a specific and still

growing expansion of those of the CTX-M type. There are also increasing reports of ESBL-producing clinical isolates expressing multidrug resistance (MDR) [11-12]. As the relationship of bacterial virulence and antimicrobial resistance still controversial and as vaginal *E. coli* is considered as a source of extraintestinal pathogenic *E. coli*, this study was performed to investigate the phylogenetic origin and extraintestinal pathogenic *E. coli* virulence factors' prevalence among well characterized ESBL-producing vaginal *E. coli* isolates from pregnant and non-pregnant women in comparison with those that are ESBL non-producers.

2. Materials and Methods

2.1. Bacterial Isolates

This study included well characterized ESBL-producing (n=40) and non-producing (n=21) vaginal *E. coli* isolates from pregnant (n=23) and non-pregnant (n=38) women, aged 18-45 years. Isolation, identification, and ESBL production and multidrug resistance of these isolates were reported in previous work [13].

2.2. DNA Preparation for PCR

Each isolate was subcultured on tryptic soy agar plate for 24 h at 37°C. From the agar plate, 5 colonies were picked and suspended in 100 µl sterile distilled water. Bacterial suspensions were run for 10 min at 94°C [14] in a DNA thermocycler (MultiGene, Labnet International, Inc., USA) and cell debris were removed by centrifugation (12,000 rpm for 1 min). Five µl of supernatant was used as a template DNA in PCR.

2.3. Phylogenetic Grouping of the Isolates

As a first step, the isolates were classified using the rapid phylogenetic grouping technique described by Clermont *et al.* [15]. This method is based on a triplex PCR involving the amplification of two genes (*chuA* and *yjaA*) and of an anonymous fragment of DNA from *E. coli*. Briefly, PCR was performed with three primer pairs, in a total volume of 25 µl containing 12.5 µl of KapaTaq 2x Ready Mix (KAPA Biosystems, USA), 20 pmol concentrations of each primer, and 5 µl of DNA template. The PCR conditions were as follows: denaturation for 4 min at 94°C, 30 cycles of 5 s at 94°C and 10 s at 59°C, and a final extension step of 5 min at 72°C.

In the second step, a multiplex PCR was performed for isolates with a standard protocol described by Clermont *et al.* [15], under the following conditions: denaturation for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and a final extension step of 7 min at 72°C.

For each isolate, these two steps were repeated at least twice and phylogeny was performed by combining the results of both protocols. The results were interpreted as follows, according to Clermont *et al.* (2000): group B2 (*chuA*+, *yjaA*+,

TspE.C2±), group D (*chuA*+, *yjaA*-, TspE.C2±), group B1 (*chuA*-, *yjaA*±, TspEC2+) and group A(*chuA*-, *yjaA*±, TspE.C2-).

2.4. Genotypic Virulence Characterization of the Isolates

Multiplex PCR was used to detect six genes encoding virulence determinants usually associated with the *E. coli* strains responsible for extraintestinal infections: *fimH* (type 1 pili), *papC* (type P pili), *sfa/foc* (type S pili and type 1C fimbriae), *hly* (alpha-hemolysin), *iucC* (aerobactin), and *neuC* (K1 capsule antigen) [16, 17]. Virulence factor genes were amplified with the primers described elsewhere [14, 18-21], in a total volume of 50 µL containing 25 µL of KapaTaq 2x Ready Mix (KAPA Biosystems, USA), 20 pmol concentrations of each primer except *hly* (30 pmol), and 5 µL of DNA template [14]. The reaction conditions were as follows [18]: initial denaturation at 94°C for 5 min followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and extension at 68°C for 3 min, followed by a final 10-min extension period at 72°C. The amplification products were separated by electrophoresis in a 2% agarose gel containing ethidium bromide. A 100-bp DNA ladder (Kappa Universal, USA) was used in each gel as a molecular size marker.

2.5. Statistical Analysis

The χ^2 test was used for statistical comparison of groups; values < 0.05 were regarded as significant [22].

3. Results

In previous work [13] we found that 40 out of 61 (65.5%) of vaginal *E. coli* isolates from pregnant and non-pregnant women were ESBL producers. In the current study these isolates were investigated using PCR-based protocols for their phylogenetic origin and virulence genotype, targeting 6 virulence genes, in comparison with ESBL non-producers. High numbers of ESBL producers and non-producers were from group B2 (47.5% vs. 42.8%, respectively) (Table 1). None of ESBL non-producers clustered in group D, whereas significant numbers ($P \leq 0.05$) of them belonged to group B1 (33.3%) compared with 20.0% and 7.5% of ESBL producers, respectively.

Significant differences in the prevalence of this study included virulence factors were not observed among isolates belonged to these two groups (Table 2).

The frequency of virulence genes according to phylogroup is shown in Table 3. In both ESBL producers and non-producers high rates of multiple virulence factors possession (three or more virulence factors/ isolate) were demonstrated among isolates belonged to groups B2 (73.6 % vs. 100%, respectively), D (50.0%, only in ESBL producers), and group A (10.0%, only in ESBL producers). While none of isolates belonged to groups A (in ESBL non-producers) and B1 had multiple virulence factors.

Table 1. Phylogroup distribution of ESBL-producing and non-producing vaginal *E. coli* isolates from pregnant and non-pregnant women.

Study Group		No. (%) of isolates positive for the indicated phylogenetic group			
		A	B1	B2	D
ESBL producers	Pregnant women's isolates (n=17)	4 (23.5)	2 (11.7)	8 (47.0)	3 (17.6)
	Non-pregnant women's isolates (n=23)	6 (26.0)	1 (4.3)	11 (47.8)	5 (21.7)
	Total (n=40)	10 (25.0)	3 (7.5)	19 (47.5)	8 (20.0)
ESBL non-producers	Pregnant women's isolates (n=6)	2 (33.3)	2 (33.3)	2 (33.3)	0
	Non-pregnant women's isolates (n=15)	3 (20.0)	5 (33.3)	7 (46.6)	0
	Total (n=21)	5 (23.8)	7 (33.3)	9 (42.8)	0
Total (n=61)		15 (24.5)	10 (16.3)	28 (45.9)	8 (13.1)

Table 2. Virulence genotypes of ESBL-producing and non-producing vaginal *E. coli* isolates from pregnant and non-pregnant women.

Study Group		No. (%) of isolates positive for the indicated virulence gene					
		<i>fimH</i>	<i>papC</i>	<i>sfa/foc</i>	<i>hly</i>	<i>iucC</i>	<i>neuC</i>
ESBL producers	Pregnant women's isolates (n=17)	15 (88.2)	8 (47.0)	4 (23.5)	6 (35.2)	12 (70.5)	6 (35.2)
	Non-pregnant women's isolates (n=23)	21 (91.3)	8 (34.7)	4 (17.3)	6 (26.0)	12 (52.1)	6 (26.0)
	Total (n=40)	36 (90.0)	16 (40.0)	8 (20.0)	12 (30.0)	24 (60.0)	12 (30.0)
ESBL non-producers	Pregnant women's isolates (n=6)	6 (100)	2 (33.3)	1 (16.6)	2 (33.3)	3 (50.0)	1 (16.6)
	Non-pregnant women's isolates (n=15)	15 (100)	7 (46.6)	5 (33.3)	6 (40.0)	6 (40.0)	3 (20.0)
	Total (n=21)	21 (100)	9 (42.8)	6 (28.5)	8 (38.0)	9 (42.8)	4 (19.0)
Total (n=61)		57 (93.4)	25 (40.9)	14 (22.9)	20 (32.7)	33 (54.0)	16 (26.2)

Table 3. Phylogenetic group distribution of virulence genes among ESBL-producing and non-producing vaginal *E. coli* isolates.

Study Group	Phylogroup (no. of isolates)	No. (%) of isolates positive for the indicated virulence gene					
		<i>fimH</i>	<i>papC</i>	<i>sfa/foc</i>	<i>hly</i>	<i>iucC</i>	<i>neuC</i>
ESBL producers (n=40)	A (n=10)	8 (80.0)	1 (10.0)	0	0	6 (60.0)	1(10.0)
	B1 (n=3)	2 (66.6)	0	0	0	1 (33.3)	1 (33.3)
	B2 (n=19)	18 (94.4)	11 (57.8)	8 (42.1)	11 (57.8)	14 (73.6)	8 (42.1)
	D (n=8)	8 (100.0)	4 (50.0)	0	1 (12.5)	3 (37.5)	2 (25.0)
ESBL non-producers (n=21)	A (n=5)	5 (100.0)	0	0	0	1 (20.0)	0
	B1 (n=7)	7 (100.0)	0	0	0	2 (28.5)	1 (14.2)
	B2 (n=9)	9 (100.0)	9 (100)	6 (66.6)	8 (88.8)	6 (66.6)	3 (33.3)
	D (n=0)	0	0	0	0	0	0

Comparison of CTX-M-producers with non-CTX-M-ESBL-producers and ESBL-non-producers revealed that there were no significant differences among these three groups regarding the possession of all studied traits except for *papC* of which CTX-M-producers and ESBL-non-producers had significantly ($P \leq 0.05$) higher prevalence (48.3% and 42.8%, respectively) than non-CTX-M-ESBL-

producers (11.1%) (Table 4). High rates of multiple virulence factors possession were demonstrated only among isolates belonged to group B2 in both CTX-M producers (73.3%) and ESBL non-producers (100%) and group D (71.4%, only in CTX-M producers), whereas none of isolates belonged to groups A and B1 had multiple virulence factors.

Table 4. Phylogenetic group and virulence genotypes of CTX-M-producing vaginal *E. coli* isolates in comparison with non-CTX-M-ESBL-producing and non-ESBL-producing isolates.

Traits		No. (%) of isolates positive for the indicated trait		
		CTX-M producers (n=31)	Non-CTX-M ESBL producers (n=9)	ESBL non-producers (n=21)
Phylogenetic group	A	6 (19.3)	4 (44.4)	5 (23.8)
	B1	2 (6.4)	1 (11.1)	7 (33.3)
	B2	16 (51.6)	3 (33.3)	9 (42.8)
	D	7 (22.5)	1 (11.1)	0
virulence gene	<i>fimH</i>	28 (90.3)	8 (88.8)	21 (100)
	<i>papC</i>	15 (48.3)	1 (11.1)	9 (42.8)
	<i>Sfa/foc</i>	6 (19.3)	2 (22.2)	6 (28.5)
	<i>hly</i>	9 (29.0)	3 (33.3)	8 (38.0)
	<i>iucC</i>	21 (67.7)	3 (33.3)	9 (42.8)
	<i>neuC</i>	9 (29.0)	3 (33.3)	4 (19.0)

Table 5. Phylogenetic group and virulence genotypes of multidrug resistant ESBL-producing and non-producing vaginal *E. coli* isolates.

Traits		No. (%) of isolates positive for the indicated trait		
		MDR-ESBL producers (n=18/32: 56.2%)	MDR-ESBL Non- producers (n=5/19: 26.3%)	Total (n=23)
Phylogenetic group	A	4(22.2)	0	4 (17.3)
	B1	1 (5.5)	0	1 (4.3)
	B2	9 (50.0)	5 (100)	14 (60.8)
	D	4 (22.2)	0	4 (17.3)
virulence gene	<i>fimH</i>	18 (100)	5 (100)	23 (100)
	<i>papC</i>	10 (55.5)	5 (100)	15 (65.2)
	<i>Sfa/foc</i>	3 (16.6)	3 (60.0)	6 (26.0)
	<i>hly</i>	5 (27.7)	5 (100)	10 (43.4)
	<i>iucC</i>	14 (77.7)	4 (80.0)	18 (78.2)
	<i>neuC</i>	7(38.8)	2 (40.0)	9 (39.1)

MDR: multidrug resistant; ESBL: extended-spectrum β -lactamase.

Multidrug resistance was higher among ESBL producers (56.2%) than among ESBL non-producers (26.3%). In total, 60.8%, 17.3%, 17.3% and 4.3% of MDR isolates clustered in groups B2, D, A and B1, respectively (Table 5). All MDR-ESBL-non producers (100.0%) belonged to phylogroup B2 compared with 50.0% of MDR ESBL-producers. The two groups did not differ significantly for all of the studied virulence factors, except for *hly* which was significantly more prevalent among all (100%) MDR-ESBL-non producers than among MDR-ESBL producers (27.7%).

4. Discussion

In previous studies [23-28] the relationship between ESBL production and bacterial virulence was extensively analyzed for ExPEC isolates from different clinical cases, specially uropathogenic *E. coli* (UPEC). As vaginal *E. coli* may be a source of clinically important ExPEC (recurrent UTI, neonatal meningitis and septicemia, and aerobic vaginitis) and as a result of unavailability of studies dealing with this subject among vaginal *E. coli*, results of this study were discussed in comparison with results of previous studies that dealt with ESBL-producing ExPEC isolates, especially UPEC and Bacteremic *E. coli*.

In total, the pattern of phylogroup distribution and virulence factor prevalence in this study included vaginal *E. coli* isolates was consistent with others [7-10]. A significant numbers of them clustered in group B2 (45.9%) (Table 1) and high percent of them showed high prevalence of ExPEC virulence genes, especially *papC*, *sfa/foc*, and *hly* (Table 2), which are characteristic virulence factors of ExPEC [14, 18]. In addition, virulence genes concentrated among isolates clustered in group B2 and to a lesser extent group D. These results confirmed the pathogenic potential of vaginal *E. coli* from both pregnant and non-pregnant women and indicated the important role played by vaginal colonization with such isolates as a reservoir for other extraintestinal *E. coli* infections [8-9].

High numbers of ESBL producers and non-producers were from group B2 (47.5% vs. 42.8%, respectively). None of ESBL non-producers clustered in group D, whereas significant numbers ($P \leq 0.05$) of them belonged to group B1

(33.3%) compared with 20.0% and 7.5% of ESBL producers, respectively. Previous studies have revealed a controversy regarding the phylogenetic distribution of ESBL-producing *E. coli*. In Turkey [29] ESBL-producing isolates distributed equally into phylogenetic groups B2 (30%), D (35%) and A (35%). Whereas others [28, 30] observed that ESBL among *E. coli* clinical strains was associated with shifts in phylogenetic distribution toward non-B2 phylogenetic groups (A/B1 and D/A, respectively). In another study [25] it was found that most ESBL-producing *E. coli* isolates were derived from phylogenetic group D (63%) whereas only 21% and 13% were from groups A and B2, respectively. These differences between our study and these studies and among these studies with each other may be due to the differences in resistance phenotype, especially ciprofloxacin resistance and ESBL type especially CTX-M subtypes [25, 30]. In our study 40.6% of ESBL producers were resistant to ciprofloxacin of which 46.1% were from group B2, compared with 23.1% of isolates clustered in each of groups D and A and only 7.6% from B1. In addition, 92.3% of ciprofloxacin resistant isolates possessed CTX-M type ESBL alone or in combination with other types. Furthermore, 38.4% of ciprofloxacin resistant isolates with CTX-M-type ESBL were from group B2. So that our results were in contrast with those reached by Johnson *et al.* [30] who concluded that ciprofloxacin resistance was associated with greatly reduced inferred virulence and shifts away from the highly virulent phylogenetic group B2. While we agreed with Pitout *et al.* [25] who demonstrated that CTX-M subtype predicts phylogenetic background. Recent reports from all over the world claimed the spread of highly virulent *E. coli* ST131 clone (mainly from group B2) producing CTX-M-15 which characterized by multidrug resistance and co-production of OXA-1 or TEM-1b β -lactamases as well as aminoglycoside resistance genes *aac(3')-IIa* and *aac(6')-Ib-cr* (which also deactivates ciprofloxacin) on transferable plasmids [31-33].

Both ESBL producers and non-producers did not differ significantly for all of this study included virulence factors (Table 2). Similar results were obtained by others [29, 34]. In this study, ESBL producers and non-producers also had similar rates of multiple virulence factors possession which was demonstrated among isolates belonged to groups B2

(73.6% vs. 100%, respectively), D (50%, only in ESBL producers) and A (10%, only in ESBL producers). These results were consistent with what is known about the concentration of virulence factors in isolates belonged to phylogroups B2 and D [35-36]. Similarly, it was reported that isolates belonging to phylogenetic groups B2 and D had more virulence factors than those belonging to A and B1, regardless of the ESBL-type they produced [26]. These virulence differences were attributed to phylogenetic differences in that the less virulent isolates were derived from low-virulence phylogenetic groups rather than because of resistance per se [23]. This suggests that the movement of the ESBL genes does not interfere significantly with the distribution of virulence factors within a particular phylogroup [37]. Hence the widespread dissemination of antibiotic resistance among bacterial populations has maintained or even increased the number of harmful bacteria involved in infections [38].

In the current work, most ESBL-producing isolates carried CTX-M-type ESBL (77.5%), all of these CTX-M ESBLs belonged to subtype CTX-M-1. Of these isolates, 51.6% clustered in group B2 versus 33.3% of non-CTX-M ESBL producers and 42.8% of ESBL non-producers (Table 4). Also, similarities were apparent between the virulence factor profiles of the isolates of these three groups, except for *papC* which was significantly ($P \leq 0.05$) more prevalent among CTX-M producers and ESBL non-producers than among non-CTX-M ESBL producers (Table 4). This *papC* prevalence difference may be attributed to the higher distribution of phylogroup B2 (in which virulence factors are concentrated) among isolates of these two groups (51.6% and 42.8%, respectively) than among non-CTX-M ESBL producers (33.3%). These findings were in agreement with Pitout *et al.* [25], Karisik *et al.* [26] and Zhang *et al.* [39], who reported that most *E. coli* with CTX-M ESBLs belonged to virulent phylogenetic groups, mainly B2 (67.0%, 60.0%, 75.0%, respectively) and the prevalence of each virulence factor was also similar irrespective of *bla*_{CTX-M} production [40]. Therefore, CTX-M production had no influence on virulence but was a major factor in clinical outcome [25]. However, this study results were in contrast with other studies [41-42] in which it was found that CTX-M-type ESBLs were mainly in *E. coli* strains with few virulence factors or in strains causing minor infections. Furthermore, 94.4% of CTX-M producers in this study were multidrug resistant of which 47.0% belonged to group B2, whereas only 26.3% of ESBL non-producers were MDR. This multidrug resistance prevalence among B2 isolates was consistent with what was obtained by Nazir *et al.* [43] where they found that group B2 was significantly overrepresented among the multidrug resistant isolates (45.4%). Similar reports were published elsewhere [26, 44-45]. This high prevalence of multidrug resistance among these highly virulent B2 group suggests that there may be features of the B2 phylogenetic type that have facilitated the acquisition of relevant resistance determinants, whether by mutation or by horizontally transmitted resistance genes. So that a particular,

multidrug-resistant, B2 clone with high fitness had arisen and spread throughout the population [43].

Worldwide, the increased distribution of multidrug resistant bacteria is the most serious point in the problem of bacterial antibiotic resistance. This property is associated with ESBL production [11-12, 46] and make treatment of infections caused by ESBL-producing bacteria more and more difficult. Here in this work prevalence of MDR was higher among ESBL producers (56.2%) than among ESBL non-producers (26.3%). As a whole 60.8%, 17.3%, 17.3% and 4.3% of this study included MDR isolates (both ESBL producers and non-producers) clustered in groups B2, D, A, and B1, respectively (Table 5). In both MDR ESBL producers and non-producers the differences were not significant for all of the studied traits except for *hly* as all MDR ESBL non-producers belonged to phylogroup B2 (in which virulence factors are concentrated) compared with only 50.0% of MDR ESBL producers. Although the differences were not significant, MDR ESBL non-producers seemed to be more virulent than MDR ESBL producers, as all of them (100%) belonged to group B2 versus 50.0% of MDR ESBL producers (Table 5). In addition, the rate of multiple virulence factors possession was higher among these isolates (100%) in comparison with 61.1% of MDR ESBL producers. In another study [23] it was reported that MDR isolates were most prevalent in group D, whereas prevalence in group B2 was the least and that the MDR isolates exhibited significantly lower prevalence of most of the screening VFs. So that the phylogenetic differences may accounted for the discrepancies between this study results and those obtained by Johnson *et al.* [23]. It was also reported that the presence of antibiotics increases the frequency of horizontal gene transfers 10- to 10,000-fold [47], thereby encouraging the horizontal transfer and recombination of virulence as well as antibiotic resistance genes [38]. These results revealed the potential pathogenicity of these isolates and required further investigation as the number of MDR non-ESBL-producers was small (only 5 isolates). These results also indicated the seriousness of infections caused by such highly virulent, MDR, ESBL-producing bacteria and stressed on the urged demand for alternative treatments of such infections and a worldwide policy to put a suitable measurements to control the spread of such MDR bacteria.

5. Conclusion

This study indicates that significant differences are not present between ESBL-producing and non-producing vaginal *E. coli* for both phylogenetic group distribution and virulence genes possession. Also, ESBL-producing vaginal *E. coli*, especially CTX-M producers, tend to be more dominant among the highly virulent phylogroup B2 and to a lesser extent group D. These data highlight the importance of vaginal colonization by these highly virulent, MDR, ESBL-producing *E. coli* as a source of extraintestinal *E. coli* infections.

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