Comparative Study of Molecular Phylogeny, Adhesion Genes and Antiobiogram of *Escherichia Coli* Clinical Isolates From High Vaginal Swabs and Urine in Women.

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Abstract

Background: *Escherichia coli* is a frequent cause of urinary tract infections, however, its identity as pathogen in the cervico-vaginal area is required to be ascertained. In addition, source (s) for *E.coli* colonzing female vagina is needed to be confirmed, whether its fecal contamination or from urinary tract.

Aim of the Study: To perform a comparative analysis of the *E. coli* clinical isolates from vagina versus those from urine in terms of molecular phylogeny, molecular determinants of virulence and antimicrobial susceptibility.

Materials and methods: A total of 60 *E. coli* strains from high vaginal swabs (n=30) and urine (n=30) were analyzed. Identification of phylogenetic groups and detection of adhesive genes were conducted by 2 different multiplex PCR systems. Antibiograms for all isolates were performed by Kirby-Bauer method.

Results and Discussion: Majority of vaginal *E coli* (VEC) isolates were belong to B2 phylogenetic group (n=20, 66.7%), whereas, majority of uro-pathogenic *E. coli* (UPEC) isolates were distributed between two phylogenetic groups, namely B2 12 (40%) and D 11 (36.7%). Therefore, most of the strains from both vagina and urine are belonging to pathogenic phylogenetic groups; however, they differ in prevalence of the groups. The *pap* gene has a higher frequency among UPEC (n= 13, 43.3%) than in VEC isolates (n=7, 23.3%). Similarly, *sfa* gene has a higher frequency in VEC isolates (n= 20, 66.7%) than in UPEC isolates 11 (36.4%). Consequently, adhesion genes playing roles in vaginal colonization may differ from that in urinary tract .VEC strains where highly susceptible to ciprofloxacin (100%) followed by nitrofurantoin (73.3%) and nalidixic acid (70%). Whereas UPEC strains were highly susceptible to nitrofurantoin (100%) followed by nalidixic acid. Thus, it seems that cirpofloxacin is appropriate for empirical therapy in vaginal infections, whereas nitrofurantoin is more appropriate for empirical therapy in UTI.

Conclusion: Strains isolated from high vaginal swabs differ from strains isolated from urine in the prevalence of phyelogenetic groups andmolecular determinants of virulence as well as in antibiograms.

Keywords: *E. coli*, *pap*, *sfa*, *afa*, high vaginal swab, Phylogeny

Introduction

Escherichia coli is a normal intestinal inhabitant of human. Nevertheless, several *E. coli* strains are frequent cause of an array of intestinal and extra-intestinal illnesses including diarrhea, urinary tract infections, septicemia, and neonatal meningitis [(1)].

Certain virulence factors occur more frequently in urinary than in fecal isolates, suggesting that uropathogenic *E. coli* (UPEC) is different from normal intestinal inhabitants [(2)]. Although *E. coli* is frequently isolated from vaginal

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epithelium [3], it is not known whether vaginal *E. coli* (VEC) isolates is different from the intestinal inhabitants or the same. Furthermore, the precise identity of vaginal VEC as a pathogen is not clear. In addition, the source of VEC is not clearly determined, whether it is from faecal contamination or from urinary tract.

Several studies support the notion that vaginal colonization with *E. coli* is an important medical condition with serious implications. Vaginal colonization with *E. coli* have been reported in 9–28% of non-pregnant women [4] and 24–31% of pregnant women [5] and shown to be associated with several genitourinary, obstetric and neonatal complications, including pelvic inflammatory disease [6]. Vaginal colonization by *E. coli* was found to be a risk factor for very low birth weight delivery and other perinatal complications [3].

Phylogenetic studies have divided *E. coli* into four major phylogenetic groups; A, B1, B2 and D [7]. Currently, phylogenetic studies are using simple and rapid technique based on triplex PCR that uses a combination of two genes (*chuA* and *yjaA*) and anonymous DAN fragment [7]. Indeed, *chuA*, is a gene required for heme transport in enterohemorrhagic O157:H7 *E. coli* [8]; *yjaA*, is a gene identified in the complete genome sequence of *E. coli* K-12 but its function is not known yet [9]; whereas, TSPE4.C2 is an anonymous DNA fragment with unknown function. Studies reported that virulent extra-intestinal strains frequently belong to group B2 and, to a less extent, to group D (7, 10-13), as well as that most commensal strains belong to group A (7, 13). Indeed, it was reported that virulence factor expression is more common among certain genetically related groups of *E. coli* which constitute virulent clones within the larger *E. coli* population. In general, the more virulence factors a strain expresses, the more severe an infection it is able to cause [2].

The ability of bacteria to adhere to host epithelial cells is considered a necessity for the establishment of infectious diseases, mainly through expression of adhesins [14, 15]. The presence of adhesins is possibly the major determinant of the pathogenicity for uropathogenic *E. coli* (UPEC) [16]. These genes are reported to play roles in movement of *E coli* from intestinal tract to urinary bladder and vagina and, consequently colonizing these sites [17]. Among the adhesins genes are type P fimbrial (*Pap*) gene, type S fimbrial adhesion gene (*sfa*), and the afimbrial adhesion gene (*afa*). Previous studies have shown that operons *pap*, *sfa*, and *afa* are prevalent in *E. coli* strains associated with urinary tract infections (pyelonephritis) in humans [18, 19]. In addition, the prevalence of adhesin genes was shown to differ between UPEC and fecal commensal strains of *E. coli* [20].

In studying the maternal carriage of extended-spectrum betalactamase-producing *E coli* isolates, ESBL-producing *E. coli* were prevalent in pregnant women [21]. Studies from worldwide have reported isolation of drug resistant E. coli among vaginal isolates of pregnant women [21-23]. Transmission of these resistant strains to the neonate can prove fatal in whom early detection is challenging and treatment options are limited. Outbreaks in neonatal wards and adverse outcome due to drug resistant E. coli infection have been reported [24, 25]. Thus identification and elimination of these resistant strains at the maternal level can have an impact on the reduction of fatal outcome in neonates especially in developing countries where the neonatal mortality rate is high [26].

The overall aim of this study was to compare the *E.coli* isolates from genital tract and from urinary tract in terms of phylogeny, virulence and antibiotic susceptibility in order to shed light on the possible source of vaginal colonization/ infection.

Materials and methods

E. coli clinical isolates

A total of 60 *E.coli* non-duplicated clinical isolates were included in this study (30 UPEC and 30 VEC). The isolates were collected over a period from December 2013 and June 2014 and all patients were attendants of the Gynaecology and Obstetrics teaching Hospital in Kerbala, Iraq. For UPEC isolates, clean midstream urine specimens from about75 female patients with urinary tract infections were collected and processed by standard microbiological isolation and identification of *E. coli* [27]. Regarding the VEC isolates, high vaginal swabs and/or endocervical swabs from 60 patients suffering from vaginal discharges and the swabs were processed for isolation and identification of *E coli* using standard microbiological techniques [27].

The determination of *E.coli* phylogenetic groups was performed by multiplex PCR as described by Clermont, *et al.* [7]. Details of primer sequences and predicted sizes of the amplified products are shown in table 1. results interpretation are summarized in Table 1. Each reaction was carried out by using a 20 µl mixture containing premixed PCR components (Bioneer Inc., Korea), 20 pmol of each primer and 3 µl bacterial lysate. The PCR steps were as follows: denaturation for 5 min at 94°C, 30 cycles of 30 s at 94°C, 30s at 55°C, and 30s at 72°C; and a final extension step of 5 min at 72°C. PCR products were visualized by electrophoresis in 1.5% agarose and ethidium bromide staining.

Table 1. Primers for the PCR assays of *E. coli* phylogeny

Target gene	Primer sequence (5'–3')	Size of amplicon (bp)
ChuA	ChuA.1 GACGAACCAACGGTCAGGAT ChuA.2 TGCCGCCAGTACCAAAGACA	279
YjaA	YjaA.1 TGAAGTGTCAGGAGACGCTG YjaA.2 ATGGAGAATGCGTTCCTCAAC	211
TspE4C2	TspE4C2.1 GAGTAATGTCGGGGCATTCA TspE4C2.2 CGCGCCAACAAAGTATTACG	152

The phylogenetic grouping of *E.coli* isolates was made on the basis of the presence of specific PCR-amplified fragments as follows(7):

- group A: (chu A - , yja A +/-, TspE4C2 -)
- group B1: (chu A -, yja A+/-, TspE4C2 +)
- group B2: (chu A+, yja A +, TspE4C2 +/–)
- group D: (chu A+, yja A -, TspE4C2 -/+)

Specific primers were used to amplify sequences of the *papC* (coding for P fimbriae), *sfa/foc* (coding for S fimbriae), and *afa* (afimbrial adhesin) operons as previously described [28]. Details of primer sequences and predicted sizes of the amplified products are summarized in Table 2. Each reaction was carried out by using a 20 µl mixture containing premixed (Bioneer Inc., Korea), 20 pmol of each primer and 3 µl bacterial lysate.

The PCR steps were as follows: denaturation for 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 65°C, and 30 s at 72°C ; and a final extension step of 5 min at 72°C. PCR products were visualized by electrophoresis in 1.5% agarose and ethidium bromide staining.

Table 2. Primers for the PCR assays of *E. coli* adhesive genes

Virulence factor	Target gene	Primer sequence (5'–3')	Size of amplicon (bp)
P fimbriae	*papC*	pap1 GACGGCTGTACTGCAGGGTGTGGCG pap2 ATATCCTTTCTGCAGGGATGCAATA	328
S and FIC fimbriae	sfa/foc region	Sfa1 CTCCGGAGAACTGGGTGCATCTTAC Sfa2 CGGAGGAGTAATTACAAACCTGGCA	410
Afa adhesins	afa	afa-f CGGCTTTTCTGCTGAACTGGCAGGC afa-r CCGTCAGCCCCCACGGCAGACC	672

Results

Figure 1 represents a sample of phylogenetic determination of *E. coli* strains by multiplex PCR, whereas Figure 2, shows representative sample of results for detection of adhesive genes by multiplex PCR. Phylogenetic groups were assigned according to the patterns generated by results of all 3 gene segments and as described previously [7].

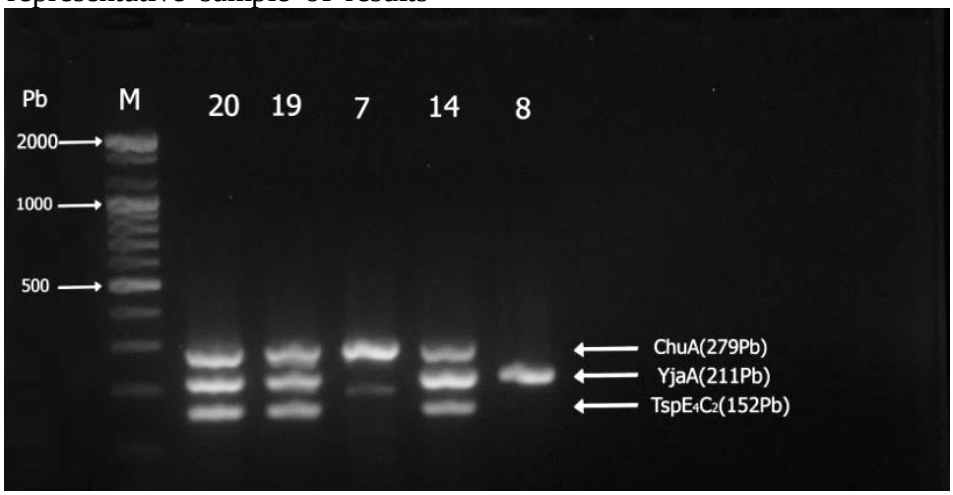

Figure 1. Agarose gel electrophoresis of *E.coli* phylogenic group genes (*chu* A, *yja* A and DNA fragment *TSPE4.C2*) detected by multiplex PCR in 60 isolates of *E. coli*. **Lane** (M), DNA molecular size marker (100-bp ladder). **Lanes** (8) group B_2 isolates showing amplification product of *yja* A (211bp) and negative result with all products of phylogenic groups respectively. **Lanes** (20),(19) and (14) group B2 isolates showing amplification products of *Chu* A and *Yja* A and *Chu* A, *Yja* A and *Tspe4.C2* (279 bp, 211 bp and 152bp) respectively.

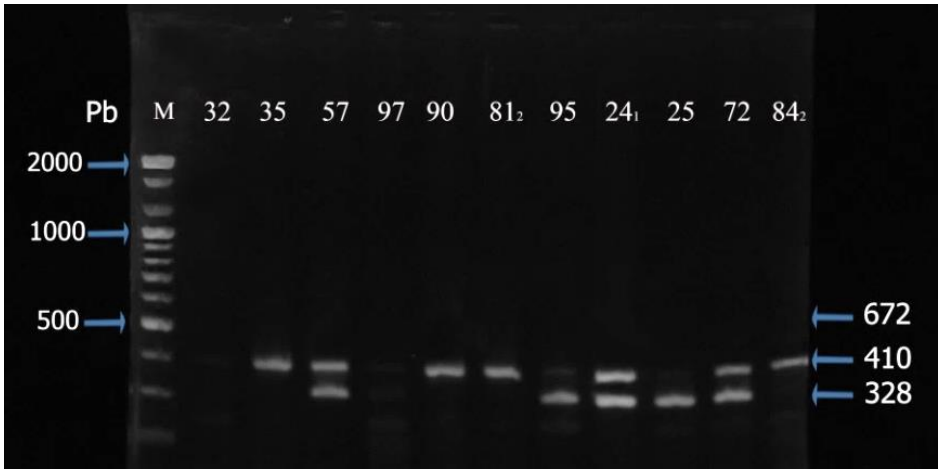

Figure 2. Agarose gel electrophoresis of *E.coli* virulence genes (*papc*, *sfa* and *afa*) genes detected by multiplex PCR. **Lane** (M), DNA molecular size marker (100-bp ladder). **Lanes** (57), (24_1), and (72) show positive results with *papc* and *sfa* virulence factors genes. **Lanes** (32) and (97) show negative results with all virulence genes. All **Lane** *E. coli* shows negative result with *afa* gene (672bp) only.

Prevalence of Phylogenetic groups and adhesins genes

Table 3 summarizes the the prevalence of the phylogenetic groups among the VEC and UPEC isolates. In this study, a highly significant difference (*P*= 0.002) was seen between VEC and UPEC isolates regarding the distribution of the phylogenetic groups. The phylogenetic group B2 was the most frequent among the strains from both vaginal and urine isolates, however, the rate of B2 group was higher in VEC isolates (66.7%) in comparison to UPEC isolates (40.0%). Importantly, 11 strains from the UPEC were phylogenetic group D vs. 1 strain from VEC and, in contrast, 8 strains from the VEC were phylogenetic group A vs. 1 strain from UPEC.

Table 3. Prevalence of Phylogenetic groups

		VEC isolates	UPEC isolates
Phylogenetic group	A	8 (26.7%)	3 (10%)
	B1	1 (3.3%)	4 (13.3%)
	B2	20 (66.7%)	12 (40%)
	D	1 (3.3%)	11 (36.7%)
	Chi-sqaure	0.002*	

*Highly significant difference. VEC, vaginal *E. coli* isolates. UPEC, uropathogenic *E. coli* isolates

Table 4 summarizes the prevalence of the adhesion genes among the VEC and UPEC isolates. Although type P fimbrial gene (*Pap*) gene was present in higher rates among UPEC isolates (43.3%) compared to VEC (23%), this difference was not significant (*P*= 0.085). On the other hand, statistically significant difference (*P*= 0.019) was noted between VEC and UPEC isolates regarding the type S fimbrial adhesion gene (*sfa*). *sfa* was present in remarkably higher rates in VEC isolates (66.7%) in comparison to UPEC (36.4%) and that this adhesin gene was the most prevalent among the VEC isolates. The afimbrial adhesion gene (*afa*) demonstrated lower frequencies among both types of isolates; nevertheless, its frequency among VEC isolates was higher (10%) than UPEC isolates (3.3%). In addition, no diiference could be seen between both type of isolates in respect to carrying single or multiple adhesins genes.

Collectively, these results shows that *sfa* adhesin gene is the most prevalent among the VEC isolates , whereas *pap* adhesin gene was the most prevalent among UPEC.

Table 4. Prevalence of adhesins genes

		VEC isolates	UPEC isolates	Chi-square
	pap	7 (23.3%)	13 (43.3%)	0.085
	sfa	20 (66.7%)	11 (36.4%)	0.019*
	afa	3 (10%)	1 (3.3%)	NC
Adhesins genes	none	8 (73.3 %)	9 (66.7%)	0.431
	Single gene	14	17	
	multiple genes	8 (26.7%)	4 (13.3%)	

*Statistically significant. VEC, vaginal *E. coli* isolates. UPEC, uropathogenic *E. coli* isolates. NC, not calculated because, numbers of some groups not fit with statistics.

Carrying virulence genes within phylogenetic groups

As shown in table 5, group B2 was the most associated with adhesins genes, however, the type of the adhesins gene was different according site from which the isolates were recovered. Among B2 of VEC isolates, 85% were carrying *sfa* gene, whereas only 54.6% of UPEC isolates were carrying this gene. On the other hand, 63.6% of UPEC isolates of B2 group were carrying *pap* gene and this is higher than in VEC isolates that only (25%) carried this gene.

Antibiograms

Regarding the antibiotic susceptibility testing, both of VEC and UPEC isolates showed high resistance to ampicillin. UPEC demonstrated remarkably higher susceptibility rate (*P*= 0.000) to ampicillin-clavulanic acid (53.3%) in comparison to VEC isolates (0.0%). In contrast, UPEC isolates were less susceptible to ciprofloxacin (73.3%) than VEC isolates (100%) and this difference in ciprofloxacin susceptibility was highly significant (*P*= 0.006).

Table 5. Distribution of adhesins genes according to the phylogenetic groups

Phylogenetic groups	VEC isolates			UPEC isolaes		
	pap	sfa	afa	pap	sfa	afa
A	1 out of 8 (12.5%)	2 out of 8 (25%)	2 out of 8 (25%)	1 out of 3 (33.3%)	1 out of 3 (33.3%)	1 out of 3 (33.3%)
B1	0 out of 1	1out of1	0 out of 1	1 out of 4 (25%)	1 out of 4 (25%)	0 out of 4 (0.0%)
B2	5 out of 20 (25%)	17 out of 20 (85%)	1 out of 20 (5%)	7 out of 11 (63.6%)	6 out of 11 (54.6%)	0 out of 11 (0.0%)
D	1 out of 1	0 out of 0	0out of0	4 out of 12 (33.3%)	3 out of 12 (25%)	0 out of 12
Total	7	20	3	13	11	1

Higher resistance rate to nalidixic acid was detected among UPEC isolates (56.7%), compared to only 7 (6.7%) resistant to this antimicrobial among VEC isolates. However, this difference was not statistically significant (*P*= 0.091). In contrast, all UPEC isolates were susceptible to nitrofurantoin (100%), whereas, only 22 (73.3%) of the cervico-vaginal isolates were susceptible to this antimicrobial and this difference in susceptibility to nitrofurantoin was statistically significant (*P*= 0.010).

Table 6. Antibiograms of *E coli* ioslated from high vaginal swabs and urine

Antibiotic	VEC isolates			UPEC isolates			Chi-square
	S	R	I	S	R	I	
Ampicillin	0	29 (96.7%)	1 (3.3%)	2 (6.7%)	28 (93.3%)	0	0.355
Ampicillin-clavulanic acid	0	28 (93.3%)	2 (6.7%)	16 (53.3%)	8 (26.7%)	6 (20.0%)	0.000*
Ciprofloxacin	30 (100%)	0	0	22 (73.3%)	8 (26.7)	0	0.006*
Nalidixic Acid	21 (70%)	7 (23.3%)	2 (6.7%)	12 (40%)	17 (56.7%)	1 (3.3%)	0.091
Nitrofurantoin	22 (73.3%)	6 (20%)	2 (6.7%)	30 (100%)	0	0	0.010**

VEC, vaginal *E. coli.* UPEC, uropathogenic *E. coli.* S, Susceptible. R, Resistant. I, Intermediate.

*Highly significant. ** Significant.

Discussion

In this study, majority of VEC isolates were belong to B2 phylogentic group (n=20, 66.7%), whereas, majority of UPEC were distributed between two phylogentic groups, namely B2 12 (40%) and D 11 (36.7%). Owing to the fact that both B2 and D groups are pathogenic [7, 10-13], most of the isolates from both types of samples in this study could be considered as pathogenic, and especially that *E. coli* colonizing the vaginal epithelium is pathogenic albeit it differs from the UPEC strains. In addition, the difference in the distribution of the phylogenetic groups

between VEC and UPEC isolates was a highly significant (*P*= 0.002). These results may point into two important attributes, first is that, *E. coli* strains colonizing the vaginal epithelium are pathogenic and, second, the strains from vagina may be different from strains isolated from urine.

To further investigate the similarity/ dissimilarity between VEC and UPEC isolates, we studied the carrying of adhesion genes. Indeed, epidemiologic investigations have shown a good correlation between the occurrence of certain human diseases and the presence of specific virulence factors in *E. coli* [29].
In this study, we selected three adhesion genes. These genes are reported to play roles in movement of *E coli* from intestinal tract to urinary bladder and vagina and, consequently colonizing these sites [17]. Operons encoding P, S, and afa adhesins contribute to the pathophysiology of urinary tract infections, whereas genes encoding for S fimbriae is correlated with the pathogenesis of neonatal meningitis [30].

In the present study, the presence of *pap* and sfa *genes* varies between VEC and UPEC strains, where *pap* gene has a higher frequency among UPEC than in VEC isolates. Similarly, *sfa* gene has a higher frequency in VEC isolates than in UPEC isolates. These results suggest that type P fimbriae are able to promote adherence to epithelial cells of the urinary tract more than epithelial cells of the vagina and that, in contrast, the type S fimbriae are able to promote adherence to epithelial cells of the vagina more efficiently than the urinary tract. In urinary tract infections, P-fimbriae mediate the specific attachment of UPEC to kidney tissue and elicit a cytokine response in these cells [2, 31]. Nevertheless, the role of P-fimbriae in genital tract infection remains unknown.

Collectively, the results of this study show that *sfa* adhesin gene is the most prevalent among the VEC isolates, whereas *pap* adhesin gene was the most prevalent among UPEC. Furthermore, the high prevalence of *sfa* gene among VEC isolates in this study may explain the high prevalence of this gene in *E. coli* strains isolated from neonatal meningitis in other studies [30]. And this finding may give additional evidence on the role of vaginal colonization in development of neonatal meningitis. In addition, the present study confirms that VEC strains possess several virulence factors allowing vaginal and/or endocervical colonization and this gives further support to previous studies that showed that VEC strains possess several virulence factors [32, 33].

In the current study, only one strain of UPEC possess *afa* gene vs. 3 VEC strains. Previous studies showed that Afa/Dr fimbrial adhesins contributed to the ability of UPEC isolates to colonize and persist long term within the urinary tract and therefore more likely to cause the recurrence of UTI episodes [34, 35].
Studying the antibiograms of VEC and UPEC isolates has demonstrated two important findings. First finding is that *E coli* isolates colonizing vaginal epithelium are drug resistant and may comprise a risk factor especially for the neonates during delivery. Second finding is that there are differences in resistance patterns between VEC and UPEC isolates. The latter findings may have several implications, such as that supporting the hypothesis that *E* coli colonizing the vagina are different from UPEC, and treatment appropriate for UPEC is not necessarily effective against VEC isolates.

In this study, VEC strains where highly susceptible to ciprofloxacin (100%) followed by Nitrofurantoin (73.3%) and Nalidixic acid (70%). Whereas UPEC strains were highly susceptible to nitrofurantoin (100%) followed by Nalidixic acid. Thus, it seems that Cirpofloxacin is appropriate for empirical therapy in vaginal infections, whereas Nitrofurantoin is more appropriate for

empirical therapy in UTI. Ciprofloxacin is the most commonly recommended therapy for UTIs during the last 10 years [36-38]. However, this study may indicate a decline in the susceptibility rate to this antibiotic among UPEC strains.

In conclusion, most of the strains from high vaginal swab and urine were shown to belong to the pathogenic phylogenetic groups and carrying molecular determinants of virulence. However, strains isolated from high vaginal swabs differ from strains isolated from urine in type of the prevalence of the phylogenic groups and virulence factors as well as in antibiogram.

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