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Molecular phylogenetic analysis of *Enterobacter* spp. isolated from urine of patients with cystitis in Babylon province, Iraq

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Enterobacter **species are important nosocomial pathogens responsible for various extraintestinal and intestinal infections. Both *Enterobacter cloacae* and *Enterobacter aerogenes* can cause hospital acquired and community acquired urinary tract infections. The aim of this study was to determine the phylogenetic analysis of *Enterobacter* spp. isolates. A total of 24 *Enterobacter* local isolates (9 isolates were *E. cloacae* (EC) and 15 isolates were *E. aerogenes* (EA) recovered from urine samples of patients with cystitis and subjected for PCR to determine the phylogenetic groups and subgroups by targeting two gene chuA, yjaA and anonymous DNA fragment TspE4.C2.The results found that the most isolates of *Enterobacter* spp. belong to the phylogeny group B2, 17(70.84%) and the largest subgroups was $B2_3$ (12/17) followed by subgroup $B2_2$ (5/17). The second phylogenetic group was group A, 4(16.66%) in which subgroup A_0 compile (3/4) and A_1(1/4). Group B1 compile 2(8.34%) followed by group D 1(4.16%). This study was the first to determine the phylogenetic groups of *Enterobacter* spp. isolates and demonstrate that these bacteria can be assigned to one of the main phylogenetic groups. Our results revealed that, Phylogenetic group B2(especially subgroup B23) was predominant among *Enterobacter* spp. isolates recovered from patients with cystitis.**

Key words: Phylogeny, cystitis, *Enterobacter* spp., Polymerase chain reaction.

Enterobacter species, particularly *Enterobacter cloacae* and *Enterobacter aerogenes*, are important nosocomial pathogens responsible for various infections [1]. They colonize the gastrointestinal (GI) tract and are an important cause of nosocomial and opportunistic infection. Wide range of Extraintestinal infections can be caused by *Enterobacter* species including bacteremia, urinary tract infections(UTIs), lower respiratory tract infection, endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, and ophthalmic infections [2,3,4]. *Enterobacter* species can also cause various community-acquired infections, including UTIs, skin and soft-tissue infections, and wound infections [5]. The source of infection may be endogenous (via colonization of the skin, gastrointestinal tract, or urinary tract) or exogenous, resulting from the ubiquitous nature of *Enterobacter* species [6,7,8].

Regarding to *Enterobacter*, the phylogenetic groups not studied until yet. Phylogenetic analysis has shown that *E. coli* strains can be assigned to one of the main phylogenetic

groups (A, B1, B2, and D)[9]. Groups A and B1 are sister groups whereas group B2 is included in an ancestral branch[10,11,12]. The distribution of a range of virulence factors thought to be involve in the ability of a strain to cause diverse diseases also varies among strains of these phylogenetic groups indicating a role of the genetic background in the expression of *E. coli* virulence[13]. The genome size differs among phylogeny groups, with A and B1strain having smaller genomes than B2 and D[14]. Phylogenetic trees of housekeeping gene sequences from the *E. coli* reference collection indicated that group diverged first and that groups A and B1 are sister groups that separated later. More recent analysis suggests that perhaps B2, rather than D is ancestral[15].

The source of *E. coli* according to phylogeny groups may classify into intestinal or extraintestinal. The extraintestinal pathogenic strains usually belongs to groups B2 and D, the commensal strains belong to groups A and B1 whilst the intestinal pathogenic strains belong to groups A, B1 and D[16]. It has developed a polymerase chain reaction (PCR) based method to characterize the phylo-groups using genetic markers: *chuA* (a gene required for hem transport in enterohemrrhagic O157:H7 E. coli)[17,18], *yjaA* (gene encodes an uncharacterized protein and is a known housekeeping gene in *E. coli* K-12, but its function has not yet been determined and TspE4.C2(an anonymous DNA fragment that has been recently identified as part of a putative lipase esterase gene [17,19].

The aim of this study was to investigate the phylogenetic groups and subgroups of *Enterobacter* spp. isolated from urine of patients with cystitis in Babylon province, Iraq using a molecular primer.

MATERIALS AND METHODS:

Samples:

One hundred five urine samples were collected from patient with cystitis who were admitted to Urology consultant clinic of Al-Hilla Surgical Teaching Hospital in Babylon city (Iraq) during the period from April 2011 to July 2011.

Bacterial cultures:

Only 24 *Enterobacter* spp. isolates (9 isolates were *E. cloacae* (EC) and 15 isolates were *E. aerogenes* (EA) recovered from urine samples who processed on MacConkey and Eosin methylene blue agar and were incubated at 37°C overnight. The identification *Enterobacter* spp. were performed by standard biochemical methods [20,21].

DNA extraction form *Enterobacter* spp.:

Genomic DNA was extracted from the *Enterobacter* spp. isolates according to instruction provided by manufacturer using Wizard Genomic DNA purification kit supplemented by (Promega, USA). The isolated DNA was checked by 0.7% agarose gel electrophoresis and viewed using UV-transilluminator.

Detection of phylogenetic groups by PCR:

PCR was conducted to determine the phylogenetic grouping of the isolates by targeting two genes, *chuA, yjaA* and anonymous DNA fragment *TspE4.C2*[17]. Each 20 µl of PCR reaction mixture for PCR contained 3µl of upstream primer, 3µl of downstream primer, 4µl of free nuclease water, 5 µl of DNA and 5µl of master mix powered in 0.2ml thin walled PCR tube. Thermal cycler used in this study was (Clever Scientific / UK). The Thermal cycler conditions were as follows: 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 30 s. A final extension of 72°C for 7 min was performed at the end of PCR. The primers used were chuA, yjaA and TspE4.C2 which generated 279, 211 and 152 bp fragment respectively. The data of the three amplification resulted in assignment of the

isolates to phylogenetic groups and subgroups as follows: subgroup A0 (group A), chuA-, yjaA-, TspE4.C2-; subgroup A1 (group A), chuA-, yjaA+ TspE4.C2-; group B1, chuA-, yjaA-, TspE4.C2+; subgroup B22 (group B2), chuA+, yjaA+, TspE4.C2-; subgroup B23 (group B2), chuA+, yjaA+, TspE4.C2+; subgroup D1 (group D), chuA+, yjaA-,TspE4.C2- and subgroup D2 (group D), chuA+, yjA-, TspE4.C2+[22,23,24]. The PCR amplification product was visualized by electrophoresis on 1.5% agarose gels for 45 min at 72 volt. The size of the amplicon was determined by comparison to the 100 bp allelic ladder (Bioneer / Korea) (Table 1).

Table 1 Primers of phylogenetic groups used in PCR

Gene	Primer sequence (5 -3)	Size (bp)	Reference
chuA F	GACGAACCAACGGTCAGGAT	279	17
chuA R	TGCCGCCAGTACCAAAGACA		
yjaA F	TGAAGTGTCAGGAGACGCTG	211	
yjaAR	ATGGAGAATGCGTTCCTCAAC		
TspE4C2 F	GAGTAATGTCGGGGCATTCA	152	
TspE4C2 R	CGCGCCAACAAAGTATTACG		

Results

A total of 24 *Enterobacter* spp. isolates ,9 isolates were *E. cloacae* and 15 isolates were *E. aerogenes*, were recovered from urine samples and subjected for PCR to determine phylogenetic groups and subgroups and to investigate the source of these isolates. The phylogenetic groups of *Enterobacter* spp. isolated from urine samples were detected by identifying the presence of specific PCR amplicon for (*chuA, yjaA, and TspE4.C2*). Group A and B1 were intestinal groups while group B2 and D were extraintestinal. chuA marker was found in all extraintestinal group isolates (Figure 1). In intestinal groups, the yjaA marker was found in group A (Subgroup A_1 only) while found in both subgroups (Subgroup $B2_2$ and Subgroup $B2_3$) of B2 extraintestinal group (Figure 2). TspE4.C2 marker was found in group B1(intestinal group) , group B2 (only in Subgroup $B2_3$) and group D (only in Subgroup D_2) (Figure 3).

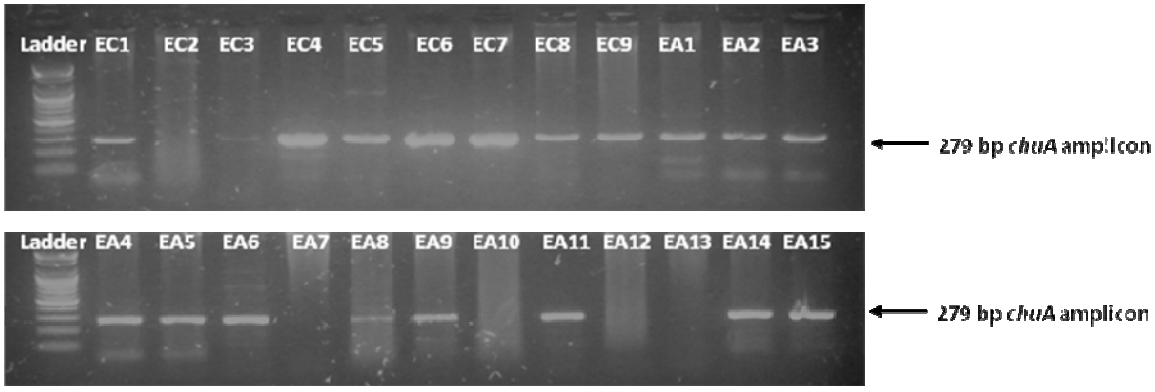

FIG 1. 1.5% Agarose gel electrophoresis of PCR of ***chuA*** amplicon; **Lane 1** and **14** represent ladder (100bp DNA molecular weight ladder). **Lane EC1-EA15** represent isolates (EC1-EA15)

The results revealed that, extraintestinal groups isolates were dominant among urine samples which compile 18(75%) while intestinal groups compose 6(25%) (Table 2). Among

extraintestinal isolates, 17(70.84%) of all isolates belong to B2 group which dividing into two subgroups, Subgroup $B2_3$ was preponderant and comprise (12/17) with profile (*chuA* +, *yjaA* +, *TspE4.C2*+) while the rest (5/17) belong to Subgroup $B2_2$ with profile (*chuA* +, *yjaA* +, *TspE4.C2* -) (Table 3).

Only one isolates (1/1) belong to Subgroup D_1 of group D with profile (*chuA* +, *yjaA* -, *TspE4.C2* -). Subgroup D_2 was not found among *Enterobacter* spp. isolates recovered from urine samples in this study. The intestinal isolates compile 6(25%) from which (4/6) belong to group A, 3 isolates represent Subgroup A_0 with profile (*chuA* -, *yjaA* -, *TspE4.C2* -) and one isolate belong to Subgroup A_1 with profile (*chuA* -, *yjaA* +, *TspE4.C2* -). The rest (2/6) belong to group B1 with profile (*chuA* -, *yjaA* -, *TspE4.C2* +). Only two isolates represent Group B1 (have no subgroups) among isolates belong to intestinal groups. Regarding to the gender of patients from which the urine samples were recovered, only (1/6) of intestinal isolates were isolated from male with the others (5/6) recovered from female. Among extraintestinal isolates (13/18) isolates were recovered from male while the rest, (5/18) isolated from female. The phylogenetic tree was drawn according to Clermont *et al.* (2000)[7] method. The result shows that Group A compile (16.66%) , Group B1 (8.34%) , Group B2 (70.84%) and finally Group D (4.16%). In this study the largest group was Group B2 followed by Group A , Group B1 and Group D(Figure 4).

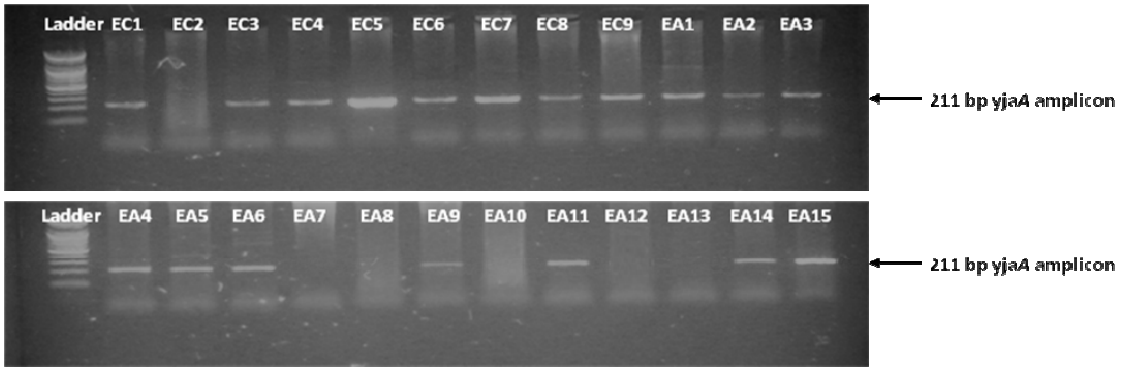

FIG 2 1.5% Agarose gel electrophoresis of PCR of ***yjaA*** amplicon; **Lane 1** and **14** represent ladder (100bp DNA molecular weight ladder). **Lane EC1-EA15** represent isolates (EC1-EA15)

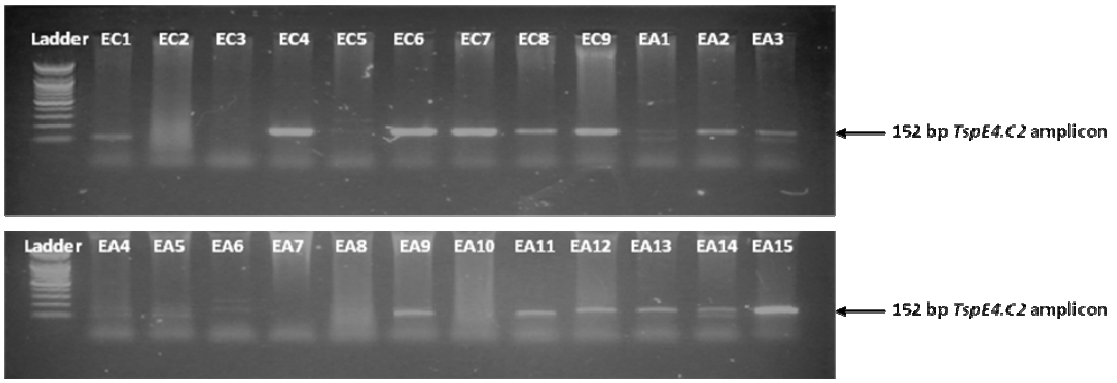

Fig. 3. 1.5% Agarose gel electrophoresis of PCR *TspE4.C2* amplicon; Lane 1 and 14 represent ladder (100bp DNA molecular weight ladder). Lane EC1-EA15 represent isolates (EC1-EA15).

Table 2 Distribution of *Enterobacter spp*. isolates according to phylogenetic groups and subgroups .

Isolate name	*chuA*	*yjaA*	*TspE4cC2*	Phylogenic group	Phylogenic subgroup	Sex of patients
EC1	+	+	+	Group B2	Subgroup $B2_3$	Male
EC2	-	-	-	Group A	Subgroup A_0	Female
EC3	-	+	-	Group A	Subgroup A_1	Female
EC4	+	+	+	Group B2	Subgroup $B2_3$	Male
EC5	+	+	-	Group B2	Subgroup $B2_2$	Male
EC6	+	+	+	Group B2	Subgroup $B2_3$	Female
EC7	+	+	+	Group B2	Subgroup $B2_3$	Male
EC8	+	+	+	Group B2	Subgroup $B2_3$	Male
EC9	+	+	+	Group B2	Subgroup $B2_3$	Male
EA1	+	+	-	Group B2	Subgroup $B2_2$	Male
EA2	+	+	+	Group B2	Subgroup $B2_3$	Male
EA3	+	+	+	Group B2	Subgroup $B2_3$	Male
EA4	+	+	-	Group B2	Subgroup $B2_2$	Male
EA5	+	+	-	Group B2	Subgroup $B2_2$	Male
EA6	+	+	-	Group B2	Subgroup $B2_2$	Male
EA7	-	-	-	Group A	Subgroup A_0	Female
EA8	+	-	-	Group D	Subgroup D_1	Female
EA9	+	+	+	Group B2	Subgroup $B2_3$	Female
EA10	-	-	-	Group A	Subgroup A_0	Female
EA11	+	+	+	Group B2	Subgroup $B2_3$	Male
EA12	-	+	+	Group B1	-	Female
EA13	-	+	+	Group B1	-	Male
EA14	+	+	+	Group B2	Subgroup $B2_3$	Female
EA15	+	+	+	Group B2	Subgroup $B2_3$	Female

Table 3 Number and percentage of *Enterobacter spp*. isolates for each phylogenetic groups and subgroups.

Phylogenic groups		Phylogenic subgroups	Sex of patients		%
			Male	Female	
Intestinal groups	Group A	Subgroup A_0 *chuA - / yjaA - / TspE4.C2 -*	0	3	16.7
		Subgroup A_1 *chuA - / yjaA + / TspE4.C2 -*	0	1	
	Group B1	- *chuA - / yjaA - / TspE4.C2 +*	1	1	8.3
Extraintestinal groups	Group B2	Subgroup $B2_2$ *chuA + / yjaA + / TspE4.C2 -*	5	0	70.8
		Subgroup $B2_3$ *chuA + / yjaA + / TspE4.C2 +*	8	4	
	Group D	Subgroup D_1 *chuA + / yjaA - / TspE4.C2 -*	0	1	4.2
		Subgroup D_2 *chuA + / yjaA - / TspE4.C2 +*	0	0	
		Total	14 (58.33%)	10 (41.67%)	100%

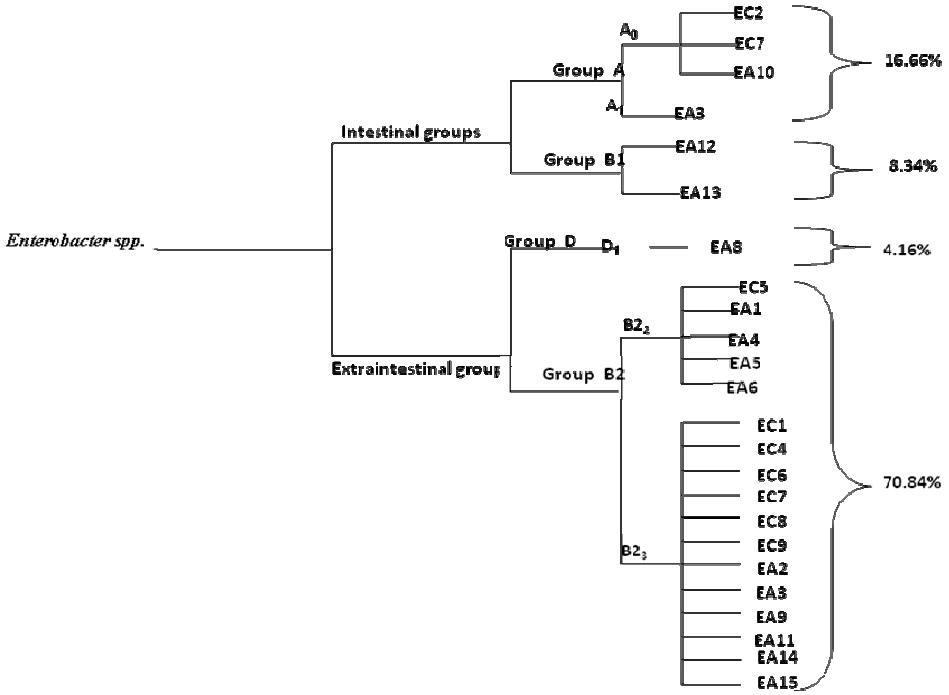

FIG 4 Phylogenic Tree of *Enterobacter spp.* isolated from urin. EC=*Enterobacter cloacae*, EA=*Enterobacter aerogenes*

Discussion

In this study the results showed that the *chuA* gene was present in all local strain isolated from urine belonging to groups B2 and D and was absent from all isolates belonging to groups A and B1[17,25]. The *yjaA* gene allowed perfect discrimination between group B2 and group D (found in all Group B2 isolates and absents in Group D isolates) and it was present in all isolates belonging to Group A (only Subgroup A_1 isolates) and absent in all isolates of group B1[22]. Also, the TspE4.C2 is present in group B1 isolates, group B2 (only Subgroup $B2_3$) and group D (Subgroup D_2) and absent from all group A isolates [17,22].

Alteri and Mobley (2007)[26] show that, ChuA is most important outer membrane proteins significantly induced in human urine as iron compound receptors. These findings show that human urine is an iron-limiting environment and concurrent production of numerous iron compound receptors by uropathogen may represent a fundamental strategy for the ability of this pathogen to colonize the human urinary tract. Many studies reveals that, *chuA* was acquired by sister groups B2 and D soon after their emergence rather than being present in common ancestor and subsequently being lost by group B1 and group D[25,27,10]. The results gathered from this study demonstrate that, among urine sample isolates the extraintestinal groups isolates were dominant while occurrence of intestinal groups was very low. This results agreed with Johnson *et al*., (2001)[27] and Zhao *et al*., (2009)[28] who found that the UPEC isolates isolated in their studies primarily belonged to one of two virulence groups group B2 or D. Predominance of B2 group among extraintestinal groups isolated from urine was in agreement with many studies[28,29]. Several recent studies suggest that extraintestinal pathogenic *E. coli* strains are mostly derived from phylogenetic group B2[16]. Among B2 group the most isolates belong to Subgroup $B2_3$ and the rest was

very little and belong to Subgroup $B2_2$. The results of our study in agreement with the results revealed by Takahashi *et al*.,(2006)[30] who demonstrate the predominance of phylogenetic group B2 isolates within the three UTI categories:(complicated cystitis(CC), complicated asymptomatic bacteriuria (CASB), and uncomplicated cystitis (UC). Many studies found similar results and revealed that, the prevalence of group B2 in the UTI isolates (83.8%) was significantly higher than that in the healthy adult stool isolates (20.0%), showing that the UTI isolates in Japan shared a similar distribution of phylogenetic groups with those strains isolated from various geographic locations such as France, the USA and the Netherlands[16,31,32]. There were no differences in the prevalence of phylogenetic groups among the UTI sources (cystitis, pyelonephritis, and prostatitis). Also, these finding are in agreement with those reported by Duriez *et al*. (2001)[33] who observed that group B2 was rare among commensal isolates whereas groups A and B1 were the most common. To our knowledge this study regard first study to determine the phylogenetic groups of *Enterobacter* spp. Our study demonstrate that, group A and B1 isolates come after group B2 among *Enterobacter* spp. isolates causing cystitis. These findings an accordance with some reports who indicate that isolates belonging to groups A and B1 can also cause disease of extraintestinal site[34]. Finally our results show that, the sex of patients with cystitis from which intestinal isolates recovered were female. This findings can be explained as retrograde ascent of bacteria from fecal bacteria via the urethra to the bladder and kidney especially in female who have shorter and wider urethra and is more readily transfer by microorganisms [35, 36, 37].

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