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Phylogenetic Grouping of Uropathogenic *Escherichia coli* Using Different Molecular Typing Methods in Kurdistan Region-Iraq

Narmin S. Merza*, Jaladet M. S. Jubrael

Scientific Research Center, Duhok University, Duhok, Iraq

Abstract

A total of 364 clinical isolates of *Escherichia coli* were collected and characterized from different hospitals in Kurdistan region. Two hundred ninety six of these were isolated from female accounting (81%) whereas 68(19%) of them were recovered from male. One hundred fifty isolates of UPEC (Fifty from each province) were randomly selected and confirmed as *E. coli* isolates at molecular level by PCR using *uidA* primer as a species-specific primer producing 670bp DNA fragment for *uidA* gene. They were assigned into one of the major phylogenetic groups (A, B1, B2 and D). This is based on the presence or absence of genetic markers including: two genes (*chuA* and *yjaA*) and a DNA fragment (TSPE4.C2) as proposed by Clermont *et al.,* 2000. It has been found that 85 (56.7%) of these isolates belonged to phylogenetic group B2, 31 (20.7%) belonged to group D, 27 isolates were phylo-group (A) a counting (18%), and only 7 isolates (4.7%) belonged to group B1. Further classification has been done in order to increase the discrimination power of *E. coli* population analyses by classification these phylogroups into seven subgroups including, A0, A1, B1, B22, B23, D1and D2. Phylogenetic group B2 showed two types of gene patterns. Seven isolates belonged to B22 and 77 isolates designated as B23. In addition to unusual strain (-++) which assigned to phylogroup B2. Phylogenetic group D revealed two gene patterns; it has been found that 11 of them were belonging to subgroup D1 and 20 isolates belonged to sub group D2. In the third phylogroup A, only 2 isolates belonged to subgroup A0 and 25 isolates belonged to subgroup A1. Out of 7 isolates which belonged to Phylogroup B1, 2 isolates had unique pattern and assigned as B1a. From these results it have been found that B23 (+++) was the most predominant among all other subgroups accounting (77/150) 51.3%, while subgroup A0 (---) was the least prevalent among all others accounting (2/150) 1.3%.

Keywords

Phylogenetic Grouping, Triplex PCR, *Escherichia coli*

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1. Introduction

Escherichia coli considered as a highly diverse bacterial species found naturally in the intestinal tract of humans and many animal species (Wiles *et al.,* 2008). Although *E. coli* is known to be part of the normal gut microbiota, there are some strains that are pathogenic causing wide variety of intestinal and extraintestinal diseases (Marrs *et al.,* 2005). Extraintestinal infections due to *E. coli* are common in all age groups and can involve almost any organ or anatomical site. Among Extraintestinal Pathogenic *E. coli* (ExPEC), strains of Uropathogenic *E. coli* (UPEC) are most commonly associated with human disease (Russo and Johnson, 2000).

These bacteria are the primary cause of community-acquired urinary tract infections (UTI) and a large portion of nosocomial UTIs, accounting for the substantial medical costs and morbidity worldwide (Foxman, 2003). In recent years, there has been an increase in the occurrence multidrug resistant strains of E. coli causing UTI and invasive infections. These organisms have particular clinical

* Corresponding author
E-mail address: narmin.merza@uod.ac (N. S. Merza)

significance due to the limited therapeutic options that are available. Furthermore, the prevention and control of the spread of uropathogenic E. coli infections hampered by a poor understanding of the population biology of these pathogens (Lau *et al.,* 2008).

The development and application of molecular diagnostic techniques has initiated a revolution in the diagnosis and monitoring of infectious diseases (Tang *et al*., 1997). Molecular tools have been introduced and founding very helpful in accurately diagnosing diseases with greater sensitivity and specificity than conventional methods. The use of such tools, especially the techniques based on polymerase chain reaction (PCR), had a huge impact on the characterization, detection, diagnosis and taxonomy of the infectious disease agents (Siripattanapipong *et al.,* 2010). PCR-based technique has been used for the identification of phylogenetic groups of the *E. coli* species and had revealed that the majority of strains belong to four phylogenetic groups: A, B1, B2, and D according to the combination of the three genetic markers chuA, yjaA and DNA fragment TspE4.C2 (Clermont *et al.,* 2000). These phylo-groups varied in their ecological niches, life-history characteristics and propensity to cause disease (Gordon *et al*; 2008). In addition to the genomic size, it has been found that the group A and B1 strains having smaller genomes than B2 or D strains (Bergthorsson and Ochman, 1998). Groups A and B1 are considered to be sister groups and group B2 is considered by some to represent the 'ancestral lineage' of *E. coli* (Lecointre *et al*., 1998). Besides, it is found that most isolates of EXEC are more likely to be B2 or D strains than to be A or B1 strains (Smith *et al.,* 2007). Thus a great deal is learnt concerning the characteristics of an unknown strain by determining its phylo-group membership (Gordon *et al*; 2008). Because of UTIs are very common in Kurdistan Region-Iraq and UPEC are the most frequent pathogens. There is a need phylogenetic grouping and subgrouping of the local isolates of UPEC collected from different hospitals in Kurdistan region-Iraq.

2. Materials and Methods

2.1. Uropathogenic *E. coli* Strain Collection

A total of 364 clinical isolates of UPEC were collected from different hospitals in Kurdistan region over a time period from December 2011 till to May 2012. This collection was carried in different settings including; at the Microbiology laboratories at the Rezgari, Teaching hospital and Azadi general hospitals in Erbil, Sulymani and Duhok provinces respectively.

2.2. Culture Media Characterization of *E. coli*

All collected clinical isolates of suspected Uropathogeneic *Escherichia coli* were recultured on selective media including; MacConkey agar, Eosin Methylene Blue agar (EMB) by streak plate method incubated at $37C^0$ for 24 hours (Cheesbrough, 2006).

2.3. DNA Extraction and *uidA* Gene Detection

DNA extraction was carried out for 150 *E. coli* isolates using commercial kit (DNP ^{TM} High yield DNA Purification). Internal fragment of *uidA* gene with molecular weight 658 bp. was amplified using pair of *uidA* primer. The amplification reaction consist of 25µl as final volume for each sample containing 12.5µl of master mix(Cinnagen-Iran), 1µl of each primer including forward and reverse (10pmol/µl), 2µl of genomic DNA (25-50ng/ µl) and 8.5µl of sterile deionized distil water. The Amplification conditions were illustrated in Table (1).

2.4. Phylogenetic Analysis

The phylogenetic group of 150 selected UPEC strains was determined according to Clermont *et al*. 2000 by Triplex PCR – analysis using two genes *chuA* and *yjaA* and the DNA fragment TspE4.C2. The amplification reaction consist of 25µl as final volume for each sample containing 12.5µl of master mix, 1µl of each primer including forward and reverse (20pmol/µl), 2µl of genomic DNA (25-50ng/ µl), and 8.5µl of sterile deionized distilled water. Amplification conditions were summarized in Table (1).

Table (1). Represents amplification conditions of Species-Specific PCR analysis using *uidA* primer and Triplex PCR analysis using *ChuA, yjaA, TSPE4C2* primers.

	Initial denaturation	Denaturation	Annealing	Extension	Final Extension	Ref.
Species-specific PCR	94C° 10min 1 cycle	92C° 1min 35cycles	58C 1min	72C° 30sec	72C° 5min 1 cycle	Adamus-Bialek *et al.*, 2009
Triplex PCR	94 C° 4min 1 cycle	94C° 5 sec 30cycles	59C° 10 sec	72C° 10sec	72C° 5min 1cycle	Clermont *et al.* 2000

3. Result and Discussion

The results of UPEC isolates incidence showed that out of 364 collected UPEC strains from three provinces (Erbil, Sulymani and Duhok) in Kurdistan Region-Iraq 296 were isolated from females counting 81% and 69 strains isolated from males

counting (19%). From the results, it became clear that the females were more susceptible to Urinary tract infections (UTIs) than males. These results were in agreement with the results of many other studies conducted. For example, it was estimated that 40-50% of women and only 5% of men will develop a UTI in their lifetime (Totsika *et al.,* 2012). Jadhav *et al.,* 2012 showed the incidence of UTI due to *E. coli*, was more in females (70%) than in males (30%). Many factors may increase the susceptibility of females to be exposed to UTI than males such as, anatomical differences and the hormonal milieu of the urinary tract (Foxman, 2003). Other reasons such as, sexual activity and contraceptive may also be included (Scholes *et al.,* 2000).

In repeated experiments, all 150 UPEC isolates were successfully amplified and they resulted in producing a single band of the *uidA* as the species specific locus in all strains with a molecular weight of about 670 bp as shown in Figure 1. The successful amplification of *uidA* amplicon in all selected samples had further confirmed at the molecular level that all these strains were in fact *E. coli*. Many studies have reported that this step is a pre-request for any further molecular investigation (Giray *et al.,* 2012). The obtained result was in agreement with a study conducted in Duhok Province using the same primer (*uidA* gene) for the identification of *E. coli* and had produced the same molecular weight (Fadel Al-Deen, 2012). However, this molecular weight was found different from those obtained by Adamus-Bialek *et al.*, 2009 with using the same primer sequence. This variation may be attributed to the fact that genes usually contain multiple coding mini- and microsatellite repeats that are highly dynamic components of genomes. Therefore, recombination events within these tandem repeats lead to changes in repeat numbers, which in turn alters the sequences. This variation may provide the functional diversity and allows rapid adaptation to the environment and/or elusion of the host immune system (Levdansky *et al.,* 2007). Since *uidA* gene is a housekeeping gene encoded *B*-D-glucuronidase enzyme (Feng *et al.,* 1991) and up to 97% of *E. coli* produce this enzyme (Daly *et al.,* 2002) therefore, many studies used this gene as a molecular marker for the identification of *E. coli* (Adamus-Bialek *et al.,* 2009, Maheux *et al.,* 2009, Giray *et al.,* 2012 and Fadel Al-Deen, 2012). This method may provide rapid and robust results in a short time for the confirmation of specific-species identification of *E. coli* and may also pave the way for further and more details molecular applications.

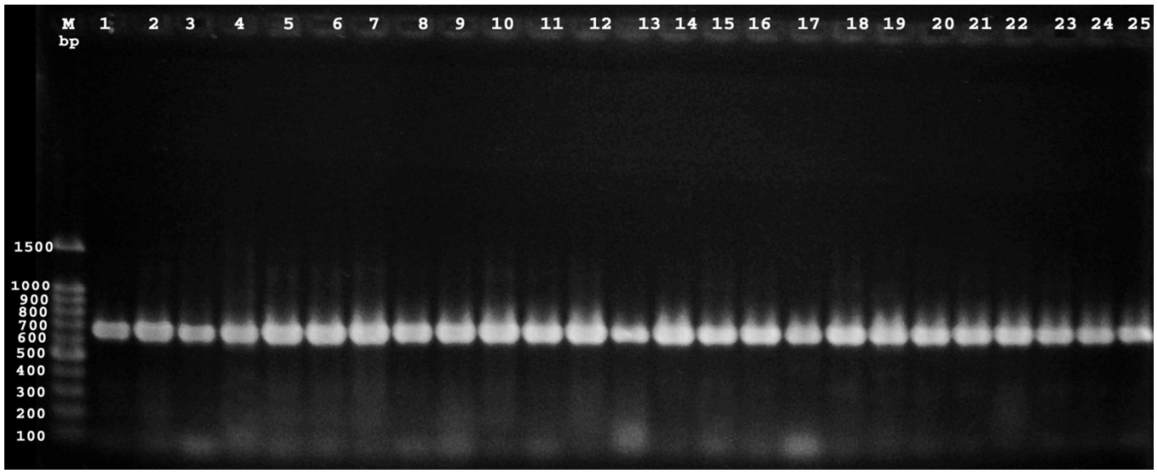

Figure (1). Represents Specific-Species PCR amplification for some UPEC strains produced with *uidA* amplicon with molecular weight 670bp. Electrophoresis was performed on (1.2%) agarose gel and run with 3V/Cm, for 2hours. Lanes M contained DNA molecular weight marker (100bp).

Triplex PCR analysis for the 150 UPEC isolates experiments were performed in order to assign them into the major phylogenetic known groups (A, B1, B2 and D) according to Clermont *et al.,* 2000 classification based on the presence (+) and/or absence(-) of the two major genes namely *chuA* and *yjaA* and TSPE4C2 fragment (DNA) markers. To increase the discrimination power of *E. coli* population analyses, these isolates were then further classified into seven subgroups including, A0, A1, B1, B22, B23, D1and D2

Figure (2) shows the different banding patterns of the Triplex PCR for samples collected from Erbil province. From this Figure, it may clearly be seen that all isolates had produced banding pattern reflecting the presence and/ or absence of the *chuA* and *yjaA* plus TSPE4C2 fragment.

The results showed that *chuA* gene was present in 41 isolates, while 34 of these isolates displayed *yja*A gene and 37 exhibited TSPE4C2 DNA fragment. The analysis of this Figure also revealed that 30 of these strains accounting 60% belonged to phylogroup B2,two of these isolates designated as subgroup B22 (++-) subgroup and 27 of them belonged to B23(+++) subgroup, in addition; to one unusual strain (sample 35) with a pattern (-++) also designated to B2 phylogroup. Thirteen of the tested isolates belonged to phylogroup D

accounting 26% which also consisted of two gene patterns. Six of these isolates belonged to D1(+--), while seven of them belonged to D2(+-+). PhylogroupA contained 5 of the UPEC isolates accounting 10% and only one of them had (---) profile named A0, whereas 4 of these isolates belonged to A1 (-+-). Finally 2 isolates belonged to B1 exhibiting a unique pattern and disgnated as B1a.

It is important to mention that strain (sample 35) which had the unique pattern characterized by the presence of *yjaA* gene *and TSPE4.C2* DNA fragment whereas it failed to amplify *chuA* gene. This unique pattern did not fit Clermont classification, however, could not be assigned to one of the seven group's pattern. Similar result, in 2007 at University Hospitals of Coimbra in Portugal, was recorded for the first time showing this combination of genes by the triplex PCR method. They had designated this *E. coli* strain (isolated from urine of a patient) as HUC270. This isolate was further subjected to antimicrobial resistance test, virulence genes profile and suggested that this strain should be assigned to the major phylogenetic group B2 (Mendonça *et al.,* 2011). Thus, this strain was also considered in this study as B2.

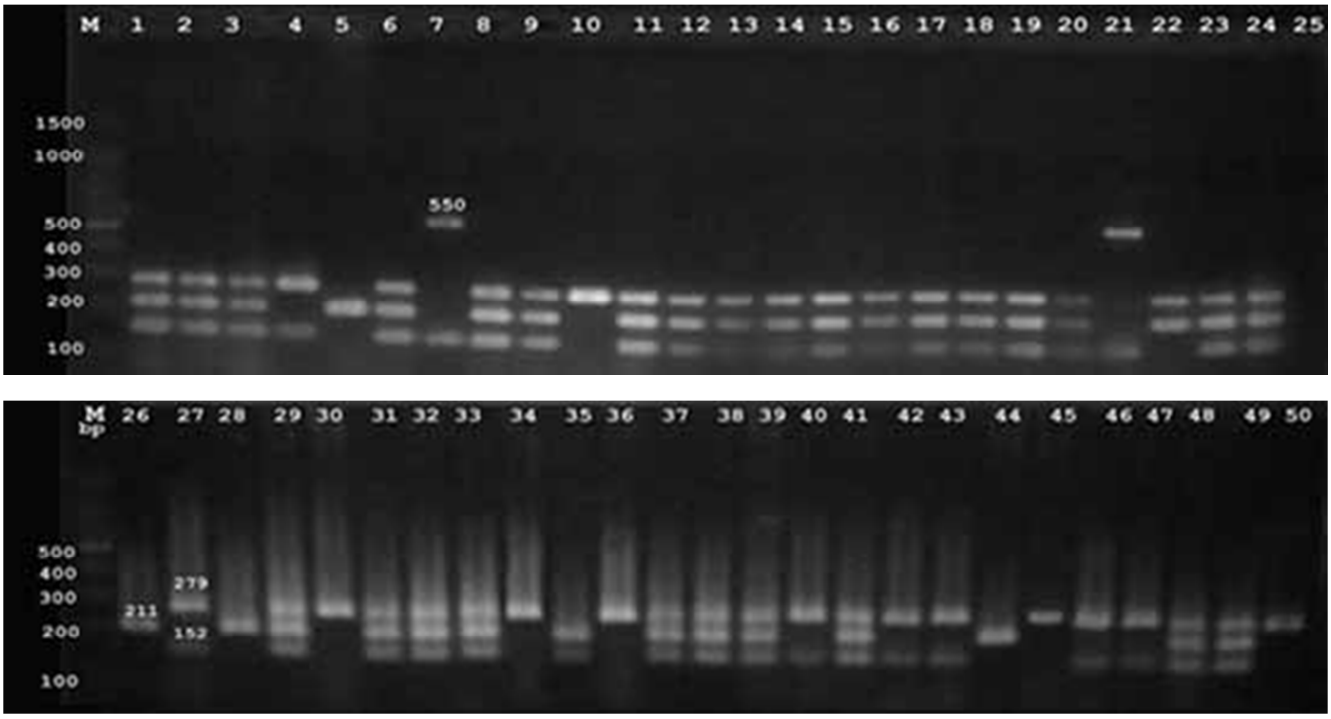

Figure (2). Represents Triplex PCR profiles specific for phylogenetic groups of UPEC strains isolated from Erbil Province. *chuA* amplicon with molecular weight 279bp., *yjaA* amplicon with molecular weight 211bp. and DNA fragment TSPE4.C2 amplicon with molecular weight 152bp. combination of these markers allowed phylogenetic group determination of a strain. Electrophoresis was performed on (2%) agarose gel and run with 3V/Cm, for 2hours. Lanes M contained DNA molecular weight marker (100bp).

Another important issue that should be mentioned in these results, is an extra band with a molecular weight of 550bp. which was observed only among phylo-group B1 (sample7 and 21) as shown in Figure (2). This extra band was found to be consistant in the repeated experiments despite modifying PCR conditions. A similar result has been reported by Ruiz *et al*., 2011, who sequenced this extra-band and found that 528bp of this fragment was 100% homologus to lipase (acetyl-hydrolase) gene found in the pathogenic strains of *E. coli* belonging to phylogenetic group B1. This report suggested that this extra band could be used to characterize a new subgroup of phylogenetic group B1 (B1a).

Figure 3 presents the DNA banding patterns of the 50 UPEC isolates collected from Sulymani province which showed to be with Clermont classification, and no unusual bands were observed. The results showed that *chuA* gene was present in 37 isolates and 39 of these isolates displayed *yjaA* gene, while TSPE4C2 DNA fragment was found in 33 isolates.

The distribution results of phylogenetic grouping and subgrouping in this province showed that 30 of these isolates belonged to phylo-group B2 accounting 60% and distributed into two main sub-groups, 4 of these isolates assigned to B22 (++-) sub-group and 26 of these isolates were assigned to B23(+++) subgroup. Among 10 isolates belonged to Phylo-groupA accounting for 20%, one isolate was assigned to A0 (---) sub-group, while other 9 isolates had A1(-+-) profile. Phylogroup D consisting of 7 isolates accounted 14% and had distributed into two subgroups, 3 isolates belonged to D1(+--) and 4 of these isolates were assigned to subgroup D2(+-+). Only 3(6%) out of 50 isolates belonged to phylo- groupB1(--+).

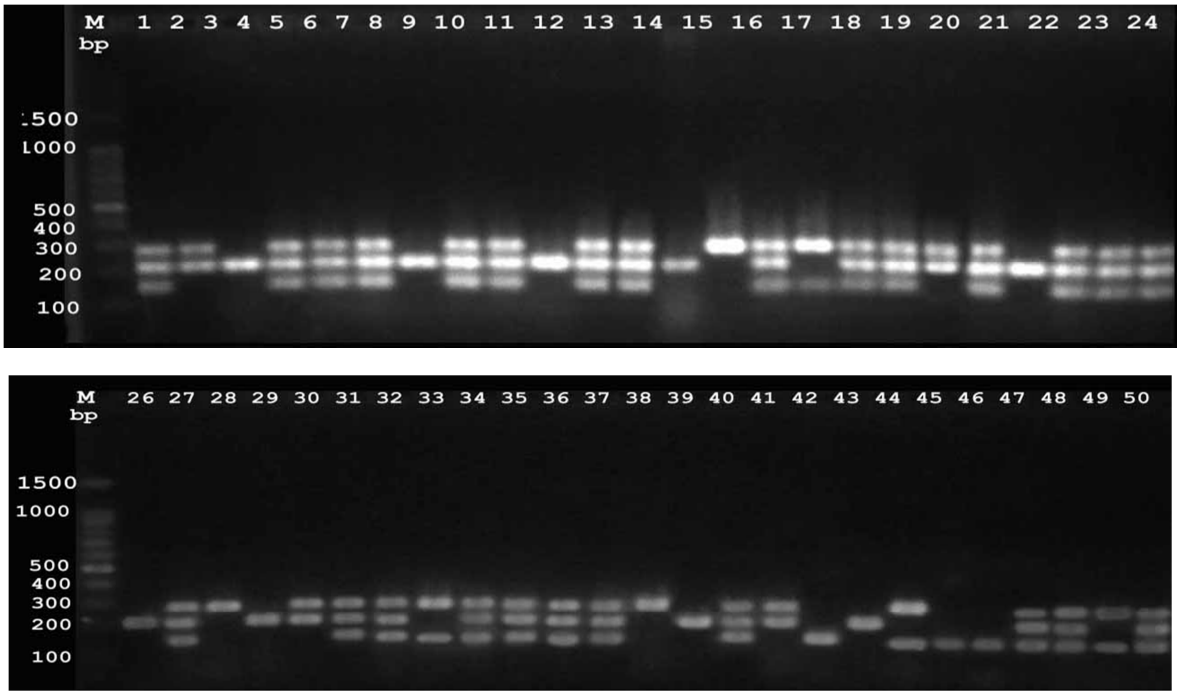

Figure (3). Represents Triplex PCR profiles specific for phylogenetic groups of UPEC strains isolated from Sulymani Province. Electrophoresis was performed on (2%) agarose gel and run with 3V/Cm, for 2hours. Lanes M contained DNA molecular weight marker (100bp).

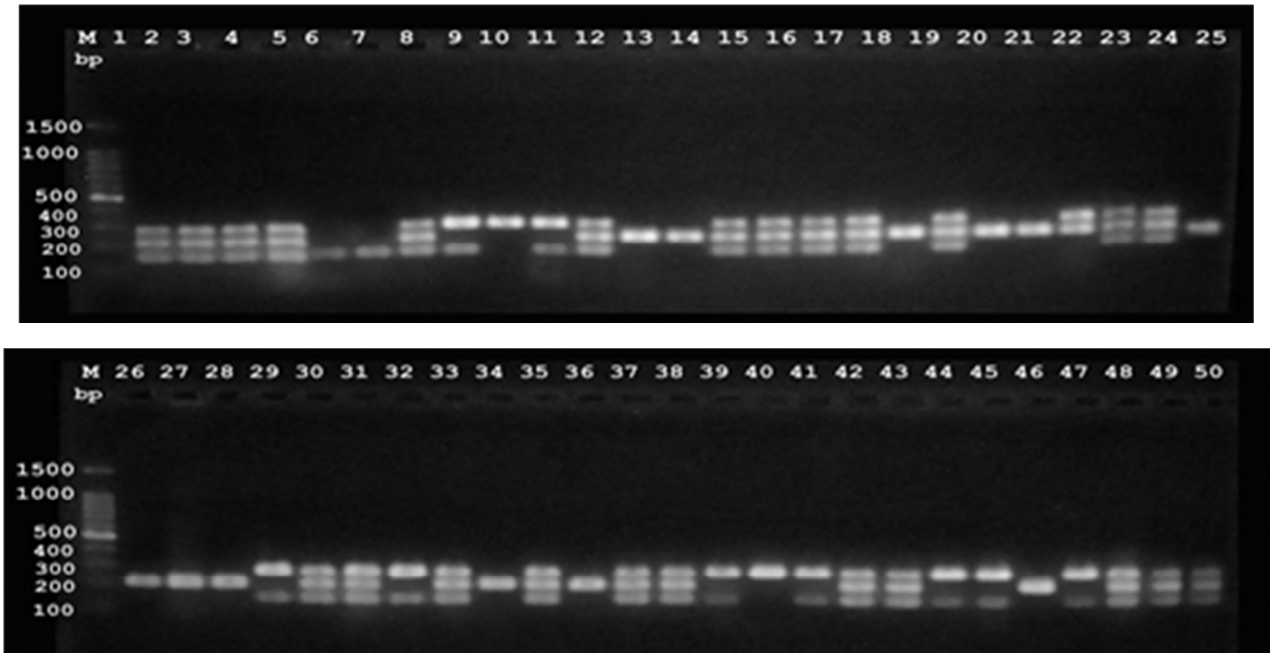

Figure (4). Represents Triplex PCR profiles specific for phylogenetic groups of UPEC strains isolated from Duhok Province. Electrophoresis was performed on (2%) agarose gel and run with 3V/Cm, for 2hours. Lanes M contained DNA molecular weight marker (100bp).

Figure 4 presents the results of the banding patterns of 50 UPEC isolates collected from Duhok province which showed to be also within Clermont classification, and they were found to be similar to those collected from Sulymani with no unusual observed bands. The results of the gene patterns in these isolates showed that *chuA* was present in 36 isolates, 37of these isolates displayed *yjaA* gene and 35 of the exhibited TSPE4C2 DNA fragment. The distribution results of phylogenetic groups and sub-groups showed that 25 of the isolates belonged to phylo-group B2 accounting 50% distributed into two main gene patterns. Only one isolate belonged to B22(++-) subgroup, while 24 of other isolates belonged to subgroup B23(+++).

Phylo-groupA consisted of 12 isolates accounting 24% and

were assigned to subgroup A1(-+-). Phylo-group D consisted of 11 isolates accounting for 22% and were distributed into two subgroups, 2 isolates assigned to D1(+--) and 9 of them belonged to D2(+-+). The final 2(4%) of UPEC strains belonged to phylo- groupB1 which had usually one gene profile (--+).

The overall results of the Triplex PCR phylo-grouping experiments in the three provinces are summarized in Table (2). From this Table it may be noticed that the highest number of the isolates were in phylo-group B2 whereas the lowest number belonged to phylo-groupB1. Subgroup B23 (+++) was found the most predominant among all other subgroups accounting 52%, while subgroup A0 (---) was the least among all others with accounting 1.3%. The phylo-group results obtained in this study were found to be in agreement with those conducted in Turkey who reported that the highest number to UPEC isolates belonged to phylo-group B2 followed by group D, then group A, and B1 (Giray *et al.,* 2012). Similar results were also reported in Iran (Navidinia *et al.* 2012). In other studies reported in Slovenia and Pakistan (Rijavec, *et al.* 2006 and Bashir *et al.* 2012) have also found that B2 was the predominant phylo-group, but with different grouping sequence (group A, then D and B1).

Table (2). Represents the distribution of phylogenetic grouping and sub grouping of 150 selected UPEC strains collected from Erbil, Sulymani and Duhok provinces in Kurdistan Region-Iraq.

Province	Phylogenetic group												
	B2				D			A			B1		
	B22	B23	Unusual	Tot.	D1	D2	Tot.	A0	A1	Tot.	B1	B1a	Tot.
Erbil	2	27	1	30	6	7	13	1	4	5	--	2	2
Sulymani	4	26	--	30	3	4	7	1	9	10	3	--	3
Duhok	1	24	--	25	2	9	11	0	12	12	2	--	2
Total	7	77	1	85	11	20	31	2	25	27	5	2	7
(%)	(56.7)				(20.7)			(18%)			(4.7)		

The predominants of subgroup B23(+++) reported in many studies and was explained by Carlos *et al.,* 2010 that this subgroup was a human source and proposed that the infections usually is caused by feces contamination. This result supports the fecal-perineal-urethral hypothesis which propose that the residing of the *Escherichia coli* strains in the rectal flora serve as a reservoir for urinary tract infections (Yamamoto *et al.*, 1997). Other studies showed that the strains of A0 (---) sub group were relatively rare and usually isolated from non-human sources such as soil, water or animals (Gordon *et al*., 2008). This may indicate that the infection caused by these strains may be due to contamination.

Another important issue to be discussed from the overall results of this study is the distribution of the two major genes (*ChuA* and yjaA) and TSPE4C2 DNA fragment which had revealed a reliable distribution between the three provinces. For example, *ChuA* gene appeared to be the most stable. It was present in almost all members of the B2 phylo-group and phylo-groupD, it was presented among 114 of 150 collected isolates accounting 76%. The stability of this gene has been attributed to the fact that it is a part of a large operon (Gordon *et al*., 2008).

The second major gene (*yjaA*) analyzed was found to be the next most stable used by the Clermont method. It was exhibited by 110 isolates accounting 73.3%, as it was all members of B2 strains and a significant fraction of phylo-groupA strains whereas it was absent in phylo-group D and B1 strains. The results of TSPE4C2 fragment distribution were the least informative of the three markers. It was displayed by 105 isolates, as it was present in all members of phylogroup B1 and the majorty of phylogroup B2 strains, whereas it was absent in the majority of D group strains and all members of phylogroup A. Gordon *et al*., 2008 distributed these three genetic markers and supposed that the genes *ChuA* and *yjaA* and the fragment TSPE4.C2 had diverse origins and they were acquired early in the *E. coli's* history and subsequently lost from some lineages.

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