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RAPID DETECTION OF UROPATHOGENIC *ESCHERICHIA COLI* VIRULENCE FACTORS IN IRAQI PATIENTS BY MULTIPLEX POLYMERASE CHAIN REACTION

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ABSTRACT

The aim of the study was to determine the occurrence of virulence genes expressing fimbriae among a hundred and twelve *Escherichia coli* isolates obtained from outpatients of seven Iraqi hospitals, between November 2014 and February 2015, showing clinical and laboratory signs of urinary tract infection (UTI).Primers to amplify the genes encoding the virulence factors of uropathogenic *Escherichia coli*, such as type 1 fimbriae (*fimH*), S fimbriae (*sfa*), pilus associated with pyelonephritis (*pap*) and afimbrial adhesin (*afa*) genes were combined to develop a multiplex polymerase chain reaction (MPCR) for detection of the respective Virulence factors and for the identification of uropathogenic *E. coli.* Among the isolates studied, the prevalence of genes coding for fimbrial adhesive systems was 91.071%, 75%, 51.785% and 5.357% for *fim H*, *sfa*, *pap* and *Afa* respectively. The various combinations of detected genes were designated as virulence patterns. A rapid assessment of the bacterial pathogenicity characteristics may contribute to a better medical approach of the patients with urinary tract infections. The multiplex PCR developed was therefore, concluded to be a useful, sensitive and rapid assay system to identify uropathogenic *E. coli.* Thus, this assay can be recommended for clinical use to detect virulent urinary *E. coli* strains, as well as for epidemiological studies.

KEYWORDS: Uropathogenic *Escherichia coli* (UPEC), Virulence factors, Multiplex polymerase chain reaction (MPCR), Urinary tract infections (UTIs), Iraq.

INTRODUCTION

6 to 90% of urinary tract infections (UTIs) in humans are caused by Gram-negative, rod-shaped, flagellated and facultative anaerobic bacterium of the family Enterobacteriaceae with name *Escherichia coli* (*E. coli*) (Cheesbrough, 2012). The UTIs comprise of a range of disorders, including cystitis (infection of the bladder) and pyelonephritis (infection of the kidney), which are defined by the presence of microorganisms like *E. coli* in urinary tract (Kulkarni *et al.*,2009). previous Investigations have indicated that the distribution of various virulence factors, such as type 1 fimbriae (*fimH*), S fimbriae (*sfa*), pilus associated with pyelonephritis (*pap*) and afimbrial adhesin (*afa*) Were useful markers for the detection of uropathogenic *E.coli* and could, therefore, be used in the diagnosis of UT1 (Yamamoto *et al.*, 1995a; Usein *et al* 2001; Tiba *et al.*, 2008; Tarchouna *et al.*, 2013; Momtaz *et al.*, 2013).

In this study, we assessed the utility of Multiplex polymerase chain reaction (MPCR) for rapid Detection of the virulence factors of Uropathogenic *E.coli* (UPEC) isolated from Iraqi patients with UTIs by a mixture of previously reported primers (Le Bouguenec *et al.*, 1992; Yamamoto *et al.*, 1995b; Struve and Krogfelt, 1999).

MATERIALS AND METHODS

Bacterial strains

A total of 112 *E. coli* strains isolated from patients of both sex and different ages presenting symptomatic UTIs were studied. Patients were visited the emergence room of seven Hospitals are AL-Muthana Military Hospital, AL-Yarmouk Teaching Hospital, AL-Karkh General Hospital, AL-Karama Teaching Hospital, AL-Imamein AL-Kadhemein Medical City, Medical City/ Teaching Laboratories and AL-Hilla Teaching Hospital in Baghdad city and Hilla city, Iraq. The strains were isolated in pure cultures and identified in the Molecular Biology Laboratory at the Institute Genetic Engineering & Biotechnology for Post Graduate Studies/University of Baghdad. Strains biochemically confirmed as *E. coli* were kept in Luria-Bertani (LB) broth/lycerol at -20°C.

DNA isolation

Bacterial strains were subcultured overnight in LB broth (Merck, Germany) and genomic DNA was extracted by using DNA extraction kit (Presto^{TM} Mini gDNA Bacteria Kit, Geneaid, Thailand) according to manufacturer's instruction.

Detection of urovirulence genes in *E. coli*

In this study, one reaction of the multiplex PCR were used for detection of four virulence factors of *E.coli* isolates from patients with urinary tract infection in Iraq. Table 1 showed the primers used for detection of UPEC virulence genes and the multiplex PCR program was comprised of the following three steps: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min. annealing at 57°C for 70s, and extension at 72°C for 70s and the final extension for 6 min at 72°C. In multiplex PCR reaction, for cycling, a DNA thermo-cycler (Eppendorf Master cycler, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) was used. A multiplex PCR was performed in total volume of 25µl and components are shown in Table 2. The amplified products were visualized by ethidium bromide staining after gel electrophoresis of 10µL of the final reaction mixture in 2% agarose (see Figuer 1).

Table 1. Primers used for detection of virulence genes in Uropathogenic *E. coli* isolates.

Gene	Primer name	Primer sequence (5'-3')	Size of product (bp)	Annealing temperature (°C)	References
fimH	fimH-F	GAGAAGAGGTTTGATTTAACTTATTG	559	58	(Struve and Krogfelt,1999)
	fimH-R	AGAGCCGCTGTAGAACTGAGG			
sfa	Sfa-F	CTCCGGAGAACTGGGTGCATCTTAC	410	55	(Le Bouguenec *et al.*,1992)
	Sfa-R	CGGAGGAGTAATTACAAACCTGGCA			
pap	Pap-F	GCAACAGCAACGCTGGTTGCATCAT	336	63	(Yamamoto *et al.*,1995b)
	Pap-R	AGAGAGAGCCACTCTTATACGGACA			
afa	Afa-F	CATCAAGCTGTTTGTTCGTCCGCCG	750	55.5	(Le Bouguenec *et al.*,1992)
	Afa-R	GCTGGGCAGCAAACTGATAACTCTC			

Table 2. Multiplex PCR reaction components.

Components	Volume (µl)
Forward primer of four genes (IDT, USA)	4 (one of each gene, con. 10 pmol/ml)
Reverse primer of four genes (IDT, USA)	4 (one of each gene, con. 10 pmol/ml)
DNA template	2
Deionized Distilled water (Bioneer, Korea)	2.5
GoTaq® Green Master Mix (Promega, USA)	12.5
volume	**25**

RESULTS AND DISCUSSION

Non- properly managed from their onset, urinary tract infection can in time, become a real threat, capable of leading to renal failure. Abetter knowledge of the virulence characteristics of the micro organism causing the infection allows the clinician to anticipate the evolution of infection in the host (Santo *et al*., 2006).

The prevalence of virulence genes ranged from 91.071% for *fimH* to 5.357% for *afa* (Table 3). Of the adhesin coding genes, *fimH* was the most prevalently detected (102 strains), followed by *sfa* (84 strains), *pap* (58 strains) and *afa* (6 strains), respectively.

Table 3. Distribution of various virulence genes of UPEC strains isolated from patients with UTIs in Iraq.

No. *E. coli* isolates	Virulence genes (%)			
	fimH	***sfa***	***pap***	***afa***
112	102 (91.071)	84 (75)	58 (51.785)	6 (5.357)

These results agree with published reports (Johnson and Stell, 2000), (Mihaylova *et al.*, 2012), (Ribero *et al* .,2008), (Tiba *et al.*, 2008), (Köves, 2014), (Ananias and Yano, 2008), (Wang *et al.*, 2002), (Momtaz *et al.*, 2013), (Usein *et al.*, 2001), (Karimian *et al.*, 2012), (Reza *et al.*, 2011), (Abass *et al.*, 2014), (Tarchouna *et al.*, 2013) and (Jadhav *et al.*, 2011), which emphasize the predominance of fimbriae type 1 among the UPEC strains. The distribution of the S fimbriae-encoding operons found among the isolates studied was higher than previously reported (Momtaz *et al.*, 2013), (Rahman and Deka, 2014), (Tarchouna *et al.*, 2013), (Tiba *et al* .,2008), (Bashir, 2010), (Oliveira *et al.*, 2011), (AL-Alak, 2012), (Santo *et al.*, 2006), (Farshad *et al.*, 2010), (Farshad and Emamghorashi, 2009) and (Arisoy *et al.*, 2006).Regarding P fimbriae, pooled results from other studies indicate that among *E.coli* isolates from patients with UTIs, approximately 80% and 30% respectively, possess P fimbriae (Johnson, 1991; Donnenberg and Welch, 1996). These results agree with our findings. A small number of UTI strains possessing *afa*-afimbrial adhesions have been reported (AL-Alak, 2012), (Bashir, 2010), (Usein *et al* .,2001), (Rahman and Deka, 2014), (Santo *et al.*, 2006), (Arisoy *et al.*, 2006), (Karimian *et al.*, 2012), (Momtaz *et al.*, 2013), (Tiba *et al.*, 2008), (Oliveira *et al.*, 2011) and (Abe *et al.*, 2008). These results agree with our findings. The results showed that there were no negative samples for virulence genes of *E.coli* isolated from Iraqi patients with UTIs.

Based on the distribution of the various targeted sequences all the studied strains exhibited 8 virulence gene patterns, referred to as E followed by an Arabic numeral (Table 4).

Table 4. Virulence patterns identified among the studied strains.

Pattern	Virulence gene				No. of strains	%
	fimH	*sfa*	*pap*	*afa*		
E1	+	-	-	-	12	10.7
E2	+	+	-	-	34	30.4
E3	+	+	+	-	40	35.7
E4	+	-	+	-	14	12.5
E5	+	-	-	+	2	1.8
E6	-	+	-	-	2	1.8
E7	-	+	+	-	4	3.6
E8	-	+	-	+	4	3.6
Total	**102**	**84**	**58**	**6**	**112**	**100%**

Two of the virulence gene patterns designated as E1 and E6 were characterized by the presence of only one gene, which was either *fim H* (12 strains) or *sfa* (2 strains). Five patterns (E2, E4, E5, E7 and E8) were represented by strain possessing a two gene association (58 strains). The patterns E3 which included strains presenting three virulencegenes were the best represented (40 strains). Among all the studied strains, there were no strains having four genes. The maximum number of detected amplicons in one strain was three virulence gene region stargeted. This result agrees with those reported by Usein *et al.* (2001) in Romania, Santo *et al.* (2006) in Brazil and Tarchouna *et al.* (2013) in Tunisia. Also, there was no simultaneous presence of *pap* gene and *afa* gene in a same strain, compatible with others (Daigle *et al.,* 1994; Santo *et al.,* 2006). Instead the presence of *afa* together with *sfa* gene and *fimH* gene was commonly detected in the same strain, as in E8 and E5 patterns, respectively. This result agrees with those reported by Blanco *et al.* (1997) and Usein *et al.* (2001) in Romania.

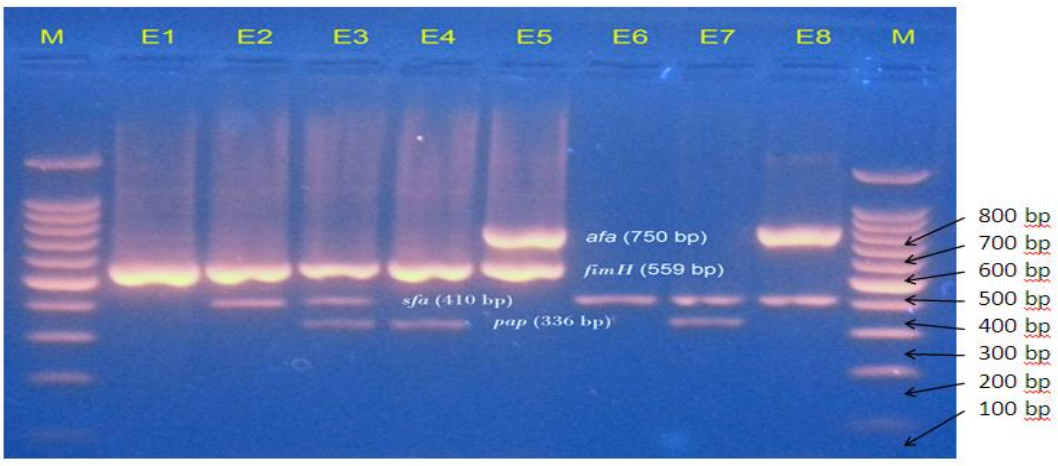

Fig.1. Detection of *fim H, sfa, pap, afa* genes by Multiplex PCR (MPCR). Lane M: DNA ladder (100 bp); Lane E1: Sample of *E. coli* isolates that contain *fim H* gene only; Lane E2: Sample of *E. coli* isolates that contain two genes (*fim H* and *sfa*); Lane E3: Sample of *E.coli* isolates that contain three genes (*fimH, sfa* and *pap*); Lane E4: Sample of *E.*

coli isolates that contain two genes (_fim H_ and _pap_); Lane E5: Sample of _E. coli_ isolates that contain two genes (_fim H_ and _afa_); Lane E6: Sample of _E. coli_ isolates that contain _sfa_ gene only; Lane E7: Sample of _E. coli_ isolates that contain two genes (_sfa_ and _pap_); Lane E8: Sample of _E. coli_ isolates that contain two genes (_sfa_ and _afa_).

A Co-dependence of these virulence factors in a particular pathogenic pathway, which may differ from that use by the *E. coli* strains expressing fimbrial adhesions and was suspected (Zhang *et al.*, 1997; Lalioui and Le Bouguénec, 2001). Some of the virulence patterns found in the studied strains could be suggestive for the presence of pathogenicity islands described in uropathogenic *E. coli* (Hacker *et al.*, 1990; Blum *et al.*, 1995). This first molecular study of *E. coli* strains isolated from Iraqi patients with UTI was meant as a step towards improving the knowledge regarding their virul encegenetic determinants. The molecular features of *E. coli* extraintestinal strains revealed by its results may contribute to a better medical approach of the patients concerned. From these results it was concluded that the multiplex PCR reported in this study is a useful methodfor the rapid concurrent detection of virulence factors and thereby the identification of *E. coli* causing UTIs.

REFERENCES

1. Abass, S.K.; Ali, M.R. and Authman, S.H. (2014). Isolation of multi antibiotics resistance Escherichia coli from urinary tract infection and the detection of PapC and fimH virulence genes by polymerase chain reaction technique. Diyala Journal for Pure Sciences, 10(1): 112-127.
2. Abe, C.M.; Salvador, F.A.; Falsetti, I.N. and Vieira, M.A. (2008). Uropathogenic Escherichia coli (UPEC) strains may carry virulence properties of diarrhoeagenic E. coli. FEMS Immunol. Med. Microbiol, 52: 397-406.
3. AL-Alak, S.K.A. (2012). Molecular Study of Adherence Factors in Uropathogenic Escherichia coli Related to Fluoroquinolones Resistance. M.Sc. Thesis. College of Science. AL- Mustansiriyah University. Iraq.
4. Ananias, M. and Yano, T. (2008). Serogroups and virulence genotypes of Escherichia coli isolated from patients with sepsis. Braz. J. Med. Bio. Res., 41(10): 877-883.
5. Arisoy, M.; Aysev, D.; Ekim, M.; Ozel, D.; Köse, S.K.; Ozsoy, E.D. andAkar, N. (2006). Detection of virulence factors of Escherichia coli from children by multiplex polymerase chain reaction. Int. J. Clin. Pract. 60: 170-173.
6. Bashir, S. (2010). Molecular Characterization of Local Human ExtraintestinalEscherichia coli Isolates. Ph.D. Thesis. Biotechnology. Quaid-i-Azam University. Islamabad. Pakistan.

7. Blanco, M.; Blanco, J.E.; Alonso, M.P. et al. (1997). Detection of pap, sfa and afaadhesin-encoding operons in uropathogenic Escherichia coli strains: relationship with expression of adhesins and production of toxins. Res. Microbiol., 148: 745-755.
8. Blum, G.; Falbo, V.; Caprioli, A. and Hacker, J. (1995). Gene clusters encoding the cytotoxic necrotizing factor type 1, Prs fimbriae and -hemolysin form the pathogenicity island II of the uropathogenic Escherichia coli strain J96, FEMS Microbiol. Lett., 126: 189-196.
9. Cheesbrough, M. (2012). District Laboratory Practice in Tropical Countries. Second edition update (part 2), Cambridge university press: India.
10. Daigle, F.; Harel, J.; Fairbrother, J.M. and Lebel, P. (1994). Expression and detection of pap-, sfa- and afa-encoded fimbrialadhesin systems among uropathogenic Escherichia coli. Canad. J. Microbiol., 40: 286-291.
11. Donnenberg, M.S. and Welch, R.A. (1996). Virulence Determinants of Uropathogenic Escherichia coliIn "Urinary Tract Infections: Molecular Pathogenesis and Clinical Management" Mobley, H. and Warren, J. (eds.), American Society for Microbiology, Washington, D.C. P: 135-174.
12. Farshad, S. and Emamghorashi, F. (2009). The prevalence of virulence genes of E. coli strains isolated from children with urinary tract infection. Saudi J. Kidney Dis. Transpl, 20: 613-617.
13. Farshad, S.; Emamghoraishi, F. and Japoni, A. (2010). Association of virulent genes hly, sfa, cnf-1 and pap with antibiotic sensitivity in Escherichia coli strains isolated from children with community-acquired UTI. Iranian Red Crescent Medical Journal, 12(1): 33-37.
14. Hacker, J.; Bender, L.; Ott, M.; Wingender, J.; Lund, B.; Marre, R. and Goebel, W. (1990). Deletions of chromosomal regions coding for fimbriae and hemolysins occur in vitro and in vivo in various extraintestinal Escherichia coli isolates, Microb. Pathog., 8: 213-225.
15. Jadhav, S.; Hussain.; Devi, A.S.; Kumar, A.; Parveen, S.; Gandham, N.; Wieler, L.H.; Ewers, C. and Ahmed, N. (2011). Virulence characteristics and genetic affinities of multiple drug resistant uropathogenic Escherichia coli from a semi urban locality in India. Plos one. A peer-Reviewed, Open Access Journal, 6(3): 1-7.
16. Johnson, J.R. (1991). Virulence factors in Escherichia coli urinary tract infection. Clin. Microbiol. Rev., 4(1): 80-128.

17. Johnson, J.R. and Stell, A.L. (2000). Extended virulence genotypes of Escherichia coli strains from patients with urosepsis in relation to phylogeny and host compromise. J. Infect. Dis., 181(1): 261-272.
18. Karimian, A.; Momtaz, H. and Madani, M. (2012). Detection of uropathogenic Escherichia coli virulence factors in patients with urinary tract infections in Iran. African Journal of Microbiology Research, 6(39): 6811-6816.
19. Köves, B. (2014). The Role of Bacterial Virulence Factors in the Clinical Course of Urinary Tract Infections. Ph.D. Thesis. Department of Microbiology, Immunology and Glycobiology. Institute of Laboratory Medicine. Lund University. Lund. Sweden.
20. Kulkarni, R.; Dhakal, B.K.; Slechta, E.S.; Kurtz, Z.; Mulvey, M.A. and Thanassi, D.G. (2009). Roles of putative type II secretion and type IV pilus systems in the virulence of uropathogenic Escherichia coli. PLoS One, 4: e4752.
21. Lalioui, L. and Le Bouguénec, C. (2001). Afa-8 gene cluster is carried by a pathogenicity island inserted into the tRNAPhe of human and bovine pathogenic Escherichia coli isolates, Infect. Immun., 69: 937- 948.
22. Le-Bouguenec, C.; Archambaud, M. and Labigna, A. (1992). Rapid and specific detection of the pap, afa and sfaadhesin- encoding operons in uropathogenic Escherichia coli strains by polymerase chain reaction. Journal of Clinical Microbiology, 30(5): 1189-1193.
23. Mihaylova, M.; Kostadinova, S. and Marhova, M. (2012). Distribution of virulence determinants and biofilm-forming among clinical urinary isolates, J. Bio.Sci. Biotech., SE/ONLINE: 45-51.
24. Momtaz, H.; Karimian, A.; Madan, M.; Dehkordi, F.S.; Ranjbar, R.; Sarshar, M. and Souod, N. (2013). Uropathogenic Escherichia coli in Iran: Serogroup distributions, virulence factors and antimicrobial resistance properties. Annals of Clinical Microbiology and Antimicrobials, 12(8): 1-12.
25. Oliveira, F.A.; Paludo, K.S.; Arend, L.N.; Farah, V.S.; Pedrosa, F.O.; Souza, E.M.; et al., (2011). Virulence characteristics and antimicrobial susceptibility of uropathogenic Escherichia coli strains. Genetics and Molecular Research, 10(4): 4114-4125.
26. Rahman, H. and Deka, M. (2014). Detection & characterization of necrotoxin producing Escherichia coli (NTEC) from patients with urinary Tract infection (UTI). Indian J. Med. Res., 139: 632-637.
27. Reza, G.; Masoud, S.; Mahmood, S.and Mahla, O. (2011). Phylogenetic background and virulence genes of Escherichia coli isolates from colisepticemic and healthy broiler chickens in Iran. Trop Anim Health Prod., 43: 153–157.

28. Ribeiro, T.M.; Yano, T. and Silva, L. D. (2008). Genotypic characterization of virulence factors in Escherichia coli strains from patients with cystitis. Rev. Inst. Med. trop. S. Paulo., 50(5): 255-260.
29. Santo, E.; Macedo, C. and Marin, J.M. (2006). Virulence factors of uropathogenic Escherichia coli from a university hospital in ribeiraopreto, Sao Paulo, Brazil. Rev. Inst. Med. trop. S. Paulo., 48(4): 185-188.
30. Struve, C. and Krogfelt, K. A. (1999). In vivo detection of Escherichia coli type 1 fimbrial expression and phase variation during experimental urinary tract infection. Microbiology, 145: 2683-2690.
31. Tarchouna, M.; Ferjani, A.; Ben-Selma, W. and Boukadida, J. (2013). Distribution of uropathogenic virulence genes in Escherichia coli isolated from patients with urinary tract infection. International Journal of Infectious Diseases, 17: 450–453.
32. Tiba, M.R.; Yano, T. and Lette, D.S. (2008). Genotypic characterization of virulence factors in Escherichia coli strains from patients with cystitis. Rev. Inst. Med. Trop. S. Paulo., 50(5): 255-260.
33. Usein, C.; Damian, M.; Tatu-Chitoiu, D.; Capusa, C.; Fagaras, R.; Daniela Tudorache, D. et al. (2001). Prevalence of virulence genes in Escherichia coli strains isolated from Romanian adult urinary tract infection cases. J. Cell. Mol. Med., 5(3): 303-310.
34. Wang, M.C.; Tseng, C.C.; Chen, C.Y.; Wu, J.J. and Huang, J.J. (2002). The role of bacterial virulence and host factors in patients with Escherichia coli bacteremia who have acute cholangitisor upper urinary tract infection. E. coli Virulence and Host Factors in AC• CID, 35: 1161.
35. Yamamoto, S.; Tsukamoto, T.; Terai, A.; Kurazono, H.; Takeda, Y. and Yoshida, 0. (1995a). Distribution of virulence factors in Escherichia coli isolated from urine of cystitis patients. Microbial. Immunol, 39: 401-404.
36. Yamamoto, S.; Terai, A.; Yuri, K.; Kurazono, H.; Yoshifumi Takeda, Y. and Yoshida, O. (1995b). Detection of urovirulence factors in Escherichia coli by multiplex polymerase chain reaction. FEMS Immunology and Medical Microbiology, 12: 85-90.
37. Zhang, L.; Foxman, B.; Tallman, P.; Cladera, E.; Le Bouguénec, C. andMarrs, C.F. (1997). Distribution of drb genes coding for Dr binding adhesins among uropathogenic and fecal Escherichia coli isolates and identification of new subtypes, Infect. Immun., 65(6): 2011-2018.