Influence of GC Content of Primer on the Number of Polymorphic Bands in Phylogenetic Analysis of Uropathogenic Escherichia coli.

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Abstract:

Fourteen serotypes of uropathogenic *Escherichia coli*, causing Urinary Tract Infection, were isolated from inpatients at Khoms University Hospital in Khoms, Libya, and subjected to biochemical, biogramatic, and phylogenetic analysis using fifteen random ten nucleotide primers. DNA amplification with each of the primers resulted in generation of different DNA fingerprinting profile with varied number of bands. Dendrogram based generation of clustering of *E. coli* serotypes showed three major

clusters, members in one of which showed most close genetic profile, and biochemical and biogramatic properties as well. Size or G+C content of the arbitrary primers did not influence number of polymorohic bands, nor does it seem to be an essential requirement for the generation of informative RAPD profiles in tested strains.

Key Words: RAPD, uropathogenic *E. coli*, UTI, Libya.

Introdction:

Escherichia coli is the most predominant pathogen causing 80-90% of community-acquired Urinary Tract Infections (UTIs) and 30-50% of nosocomially acquired UTIs. Recurrent UTIs are reported in 25% of women within 6 months of an acute UTI episode and pose a major problem (Ejrnaes, 2011[6]). Uropathogenic *E. coli* can cause also cause asymptomatic bacteriuria in catheterized patients as well (Abraham *et al.* 2012[1]).

Most UTI are asymptomatic and can be detected only by quantitative urine culture (Di- Bonaventura *et al*. 1998[5]). Fever is the most frequent finding in the symptomatic patients. Pyuria, pus in urine, is found in half of UTI patients (Lees, 1996[10]). Uropathogenic strains of *E. coli* colonize the vagina and periurethral region from where they ascend to the bladder or kidney causing severe cases of UTI, namely cystitis or pyelonephritis (Grag *et al*. 2015[7]).

Efficient methods for strain identification are required to study the epidemiology of infections caused by *E. coli,* and to screen for possible spread of *E. coli* strains in hospital wards. Traditionally, *E. coli* isolates have been characterized by serotyping with the lipopolysaccaride (O-antigen), the capsular (K-antigen), and flagellar (H-antigen) components. However, not all *E. coli* isolates can be serotyped, and strains within a

given serotype cannot several times be distinguished from one another. Accordingly, genotyping on the molecular level is considered more discriminatory than phenotypic methods and is increasingly used in diagnostic laboratories. It has been suggested that comparing data obtained by different typing methods will give an optimal insight into strain relatedness (Beatriz *et al.* 1997[2]).

Classical PCR and its various variations have been used in the diagnosis and characterization of a wide variety of organisms. One variation is randomly amplified polymorphic DNA (RAPD) PCR introduced by Welsh and McClelland (1990[17]). The distinctive DNA patterns generated by RAPD for each bacterial strain is a reflection of the genetic diversity and relatedness present among species and strains. Therefore, increasing use of RAPD-PCR in identification and characterization of various organisms has been reported to connect or disconnect sources of infection (Kumar *et al*. 2011[9]). Cave *et al*. (1994[3]) reported that RAPD technique leads to great accuracy in bacterial classification and epidemiology as compared to conventional bacteriological methods. Moreover, its simplicity and rapidity makes it particularly well adapted to many medical assays. Abraham *et al*. (2012[1]) found that many asymptomatic bacteriuria strains of uropathogenic *E. coli* were similar to catheterized asymptomatic bacteriuria strains and symptomatic UTI strains based on RAPD patterns.

This work aims to estimate genetic relatedness of *E. coli* isolates causing UTI in Khoms, Libya, by PCR analysis using randomly amplified genome segments through the RAPD technique, and evaluate effect of GC primer content on reproducible genotypic similarities. Information gained from this work would shed light onto similar sources of infection and

pattern of transmission, and hence enable us to practice effective infection control.

Materials and Mertods:

Bacterial Strains:

Midstream urine samples were obtained, in sterile containers, under aseptic conditions, from patients classified to suffer from UTI, at Khoms University Hospital, without prior administration of antibiotic treatment. 0.01 ml from mixed samples were streaked on agr plates of MacConkey and EMB, and incubated at 37°C for 24 hours.

Identification of *E. coli* was based on colonial morphology on MacConkey agar, Gram staining, differential media, and on biochemical tests included in the API-20E system (API, La Balme-les-Grottes, France). Samples with a count of >10, 000 CFU of *E. coli* per ml of urine were processed. Pure colonies were streaked on nutrient slant, labeled and incubated for 24 hr at37°C and refrigerated for later usage.

Antibiotic Sensitivity:

The Disk Diffusion Method (Kirby-Bauer) of antibiotic sensitivity testing was performed on pure isolates in accordance to National Committee for Clinical Laboratory Standards (NCCLS 1975[11]), using antibiotics and potency as shown in Table 1.

Table 1. Standard Mm distance of sensitive, intermediate, or resistant patterns of antibiotic resistance for *E. coli* according to National Committee for Clinical Laboratory Standards (NCCLS).

Antimicrobial agents	Disc potency	Resistant	Intermediate	Sensitive
Amikacin	30µg	$\geq$17	15-16	$\leq$14
Amoxicillin/Clavulanic Acid	20µg	$\geq$18	14-17	$\leq$13
Cefotaxime	30µg	$\geq$26	23-25	$\leq$22
Ciprofloxacin	5µg	$\geq$21	16-20	$\leq$15
Gentamicin	10µg	$\geq$15	13-14	$\leq$12
Nalidixic Acid	30µg	$\geq$19	14-18	$\leq$13
Trimethoprim sulfamethoxazole	25µg	$\geq$16	11-15	$\leq$10

According to biochemical and biogramatic results of the fourteen isolates, we chose the most close nine strains of classic *E. coli* isolates for RAPD analysis. A control with no DNA template was carried throughout the genetic analysis.

Genotyping of bacterial isolates:

Isolation of Bacteria DNA:

Bacterial DNA was extracted by using phenol-chloroform method as described by Cheng and Jiang (2006[4]) with some modifications. Briefly, stock cultures were inoculated into nutrient broth for 24 hr at 37°C. Three ml of each of the grown culture were centrifuged in two microfuge tube (1.5 ml each) for 2 min at 5000 rpm (Beckman S12, USA), The supernatant was then poured off. The pellet was suspended in 700µl TE buffer. Thirty µl of 20% SDS and 3 µl of Proteinase K reagents were added. Contents were incubated for 1hr at 37°C. Equal volume of Phenol/Chloroform (1:1)

was added and mixed properly but very gently to avoid DNA damage. The mixture was centrifuged for 10 min at 12000 rpm, at room temp. The upper aqueous phase was transferred to a new 1.5 ml tube, then equal volume of Phenol:Chloroform (1:1) was added. The mixture was well mixed, and spind for 10 min at 12000 rpm. The upper aqueous phase was transferred into a new 1.5 ml tube. One tenth volume of 3M Sodium acetate was added, and mixed gently. Six volumes of isopropanol was added and mixed gently until the DNA precipitated evidently. To precipitate the DNA pellet, centrifugation was performed for 10 min at 12000 rpm. DNA pellet was washed with 300 µl of 70% ethanol, followed by centrifugation for 10 min at 10000 rpm, Supernatant was decanted and the pellet was left to dry for 15 minutes. DNA pellet was resuspended in 50 µl of TE buffer, and later stored at 4°C. DNA concentration was determined using U.V spectrophotometer at 260/280 nm.

DNA quality was determined on Agarose gel as follow: Agarose gel was prepared by adding 0.8g agarose to 100 ml of 1x TAE buffer, and then boiled for 2 min in a microwave (Hommer 8PAL, N. Korea). Solution was allowed to cool to 60°C. Five µl of Ethidium bromide were added then poured on the gel in the electrophoresis tray with the special comb. Electrode tank was filled with 1XTAE running buffer. The comb was removed gently. Ten µl of each DNA sample was mixed with 2 µl of loading buffer. DNA run was performed for 1 hr at 100v. DNA quality was detected using UV transilluminator (Orion S22, USA).

DNA Analysis by RAPD-PCR

Fifteen random ten mer primers (OP) were used to amplify DNA. The sequence of used primers (OP Technology, USA) is illustrated in Table 2.

Table 2. Sequence of primers used in the RAPD-PCR

Primer	Sequence	MW
OPA-12	TCGGCGATAG	3059
OPA-13	CAGCACCCAC	2933
OPA-14	TCTGTGCTGG	3041
OPA-15	TTCCGAACCC	2939
OPA-17	GACCGCTTGT	3010
OPA-18	AGGTGACCGT	3059
OPA-19	CAAACGTCGG	3028
OPA-20	GTTGCGATCC	3010
OPO-11	GACAGGAGGT	3108
OPO-13	GTCAGAGTCC	3019
OPO-14	AGCATGGCTC	3019
OPO-15	TGGCGTCCTT	3001
OPO-17	GGCTTATGCC	3010
OPO-19	GGTGCACGTT	3050
OPO-20	ACACACGCTG	2988

PCR mixture:

PCR reaction mix contained DNA (1.5µl), dNTPs (2.0µl), primer (1.5µl), 10x buffer (2.5µl), Taq polymerase (0.5µl) and H_2O up to (25µl).

PCR conditions:

One cycle at 94°C for 3 min using New Wave thermocycler (UK) followed by Thirty five cycles as follows: 94 °C for 1 min., 37 °C for 30 seconds, and 72 °C for 2 min., followed by one cycle at 72 °C for 7 min. and one cycle at 4 °C for 7 more min.

PCR products detection:

DNA banding patterns were developed electrophoreticaly using the same steps as used to examine the genomic DNA quality but with agarose concentration of 1.2%, and detected using same UV transilluminator, Polaroid (Polaroid, UK), instant shots were gained thereafter.

Analysis of RAPD data

The bands were manually scored ‘1’ for the presence and ‘0’ for the absence and the binary data were used for statistical analysis. The scored band data (presence or absence) was subjected to cluster analysis using STATISTICA Data Miner (Statsoft, USA). The dendrogram was constructed by Ward’s method of clustering using minimum variance algorithm through the computer program IBM SPSS Statistics Base. The dissimilarity matrix was developed using Squared Euclidean Distance (SED), which estimated all the pairwise differences in the amplification product.

RESULTS:

Biochemical assay:

Fourteen different isolates of *E. coli* were collected from Khoms University Hospital. No variation among isolates in their reactions to the

first 14 tests in the API strip was seen, suggesting that these isolates are typical *E. coli* cultures as confirmed by manufacturer's profile coding of bacterial identification.

Antibiotic sensitivity

The antibiotic susceptibility of the fourteen *E. coli* isolates under investigation were assessed using the seven different antibiotics outlined in table 1.

Table 3. Biogram profile of the 14 *E. coli* isolates towards assayed antibiotics

Antibiotic	Isolate No.													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Amikacin (30µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Amoxicillin / Clavulanic Acid (30µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Cefotaxime (30µg)	R	R	I	S	R	R	R	R	I	S	R	R	R	I
Ciprofloxacin (5µg)	S	S	R	S	R	R	S	S	I	S	S	S	S	S
Gentamicin (10µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	I
Nalidixic Acid (30µg)	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Trimethoprim sulfamethoxazole (25µg)	R	S	R	S	R	R	I	R	R	R	S	I	I	S

Antibiotic sensitivity patterns. R: Resistant S: Sensitive I: Intermediate.

Variable responses were recorded for all 14 isolates towards the antibiotics ciprofloxacine, cefotoxamine, trimethorim s. methoxazole, while consistent resistance was shown against Amikacin, Amoxicillin/Clavulanic Acid and Nalidixic Acid.

RAPD Analysis:

A total of variable number of 122 bands were produced through PCR for all the nine selected isolates and the fifteen used random primers, Table 4. No bands were seen in control wells.

Table 4. Numbers of different DNA-segment bands produced by the fifteen primers applied to the nine selected *E. coli* isolates in RAPD Analysis.

Primer	Sequence	MW	Isolate No.									No. of Different Bands
			1	2	3	4	5	6	7	8	9	
OPO-11	GACAGGAGGT	3108	7	7	8	8	8	10	9	8	8	10
OPA-12	TCGGCGATAG	3059	6	6	8	7	7	7	7	8	8	9
OPA-18	AGGTGACCGT	3059	5	6	3	6	1	6	6	6	4	7
OPO-19	GGTGCACGTT	3050	4	4	4	4	4	2	4	2	3	7
OPA-14	TCTGTGCTGG	3041	10	10	6	9	7	10	7	9	4	13
OPA-19	CAAACGTCGG	3028	8	7	8	9	4	8	8	6	6	10
OPO-13	GTCAGAGTCC	3019	6	5	7	6	7	4	3	6	7	8
OPO-14	AGCATGGCTC	3019	4	3	2	3	6	6	6	4	7	8
OPA-17	GACCGCTTGT	3010	4	5	5	5	7	6	6	4	6	8
OPA-20	GTTGCGATCC	3010	5	5	6	6	6	5	5	6	5	6
OPO-17	GGCTTATGCC	3010	4	5	5	5	5	3	3	3	4	7
OPO-15	TGGCGTCCTT	3001	3	4	4	5	5	4	3	4	5	6
OPO-20	ACACACGCTG	2988	4	4	4	4	4	3	3	4	4	5
OPA-15	TTCCGAACCC	2939	7	7	8	7	8	9	9	8	8	11
OPA-13	CAGCACCCAC	2933	5	2	6	6	6	6	6	6	5	7
Total No. of Bands			82	80	84	90	85	89	85	84	84	122

RAPD profiles using primer A_{12}:

Table 5. Banding patterns of the nine selected *E. coli* isolates using primer A_{12}

Base Pairs	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9
1932	1	1	1	1	1	1	1	1	1
1591	0	0	1	1	1	1	1	1	1
1369	0	0	1	0	0	0	0	0	0
1205	1	1	1	1	1	1	1	1	1
954	1	1	1	1	1	1	1	1	1
695	1	1	1	1	1	1	1	1	1
537	1	1	1	1	1	1	1	1	1
471	0	0	0	0	0	0	0	1	1
405	1	1	1	1	1	1	1	1	1

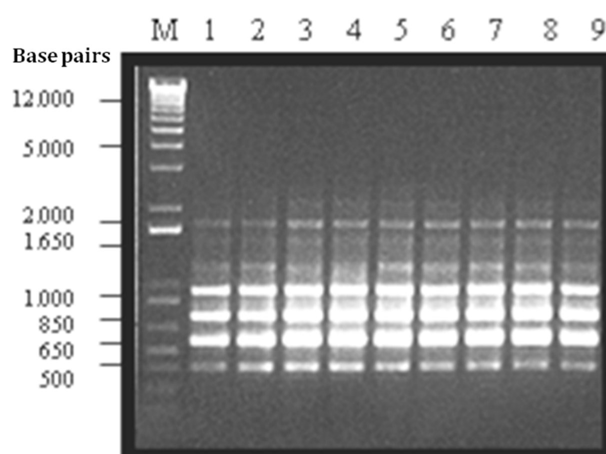

Figure 1. Gel profile study showing M (Marker) with molecular weight in base pairs and banding patterns of the nine selected *E. coli* isolates using primer A_{12}.

Table 5 shows that primer A_{12} (TCGGCGATAG, MW: 3059) produced a variable numbers of amplified bands in the nine isolates under investigation which were 6, 6, 8, 7, 7, 7, 7, 8 and 8 in the nine isolates,

respectively. Isolate No. 3 produced a unique band with 1369bp length, while it was absent in all the other eight selected isolates. Another band with 471bp length was found only in isolates 8 and 9, while it was absent in the other isolates. On the other hand, primer A_{12} produced six universal bands which were present in all of the nine isolates under investigation with lengths of 1932, 1205, 954, 695, 537 and 405bps, these universal or common bands reflect a kind of similarity between the nine *E. coli* isolates under investigation.

For both RAPD analysis banding, patterns with a difference of >2 bands were considered to represent different strains, while isolates with >2 bands difference were regarded as the same strain (Vogel *et al*. 2001[16]).

We conclude that over the fifteen different used primers and the nine *E. coli* isolates, some bands represent full similarity and other showed some variation among these isolates. Similarity index among the nine isolates and the fifteen used primers as calculated by the SPSS software are illustrated in Table 6.

Table 6. Similarity index among the nine different (varian) genotypes of *E. coli* according their RAPD profiles.

Case	VAR01	VAR02	VAR03	VAR04	VAR05	VAR06	VAR07	VAR08	VAR09
VAR01		0.911	0.87	0.867	0.739	0.887	0.817	0.797	0.793
VAR02	0.911		0.832	0.881	0.734	0.852	0.797	0.81	0.789
VAR03	0.87	0.832		0.876	0.839	0.864	0.843	0.807	0.839
VAR04	0.867	0.881	0.876		0.838	0.923	0.873	0.855	0.786
VAR05	0.739	0.734	0.839	0.838		0.843	0.821	0.748	0.759
VAR06	0.887	0.852	0.864	0.923	0.843		0.923	0.844	0.81
VAR07	0.817	0.797	0.843	0.873	0.821	0.923		0.855	0.855
VAR08	0.797	0.81	0.807	0.855	0.748	0.844	0.855		0.852
VAR09	0.793	0.789	0.839	0.786	0.759	0.81	0.855	0.852	

The two most similar isolates were No. 4 and 6 with the highest similarity value of 0.923. As well, isolates No. 6 and 7 have the same similarity value of (0.923). On the other hand, the two most varied *E. coli* isolates were No. 2 and 5, with the lower similarity value of 0.734, followed by isolates No. 1 and 5, with similarity index value of 0.739.

Genetic Relationships:

Figure 2 shows a dendrogram depicting the genetic distance among the nine *E. coli* isolates. Three main clusters were found, the first included isolates 6, 7 and 4 in a sub cluster and 3, 1 and 2 in the other sub cluster, the second main cluster included isolates No. 8 and 9. The third main cluster included isolate No. 5. The dendrogram results indicate that the most two closely related isolates were isolate No. 6 and 7, and the two most distantly related isolates were isolates No. 2 and 5. Dendrogram outline is in agreement with that of similarity index, where both of the two analyses indicate a closely related genetic make up between the two isolates 6 and 7.

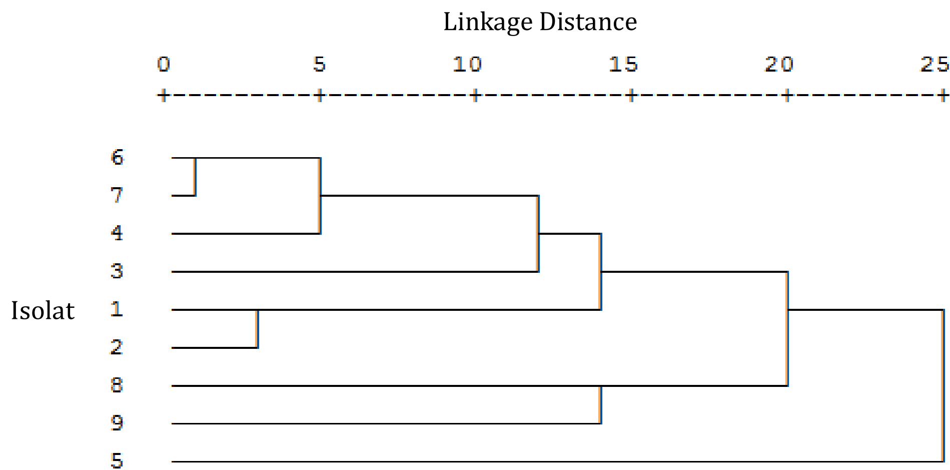

Figure 2. Dendrogram classifying the nine different genotypes of *E. coli* into groups according their RAPD profiles similarities.

Comparing dendrogram results to biogram and biochemical profiles (API20E) of the nine tested isolates, we find closely similar reaction patterns between isolates 6 and 7 throughout, thus presenting an index of closest similarity of 22% of the total nine isolates tested by RAPD.

Discussion:

To study the transmission of uropathogenic bacteria between patients, typing methods that allow for discrimination at the strain level are required; therefore this study assessed the performance of RAPD analysis of 9 strains of uropathogenic *E. coli* with unknown epidemiology.

Our results were in agreement with the finding of (Neamati *et al.* 2015[12]) who reported that 111 (74%) of the total 150 uropathogenic *E. coli* isolates were multidrug-resistant. High resistance was observed against ampicillin (81.3%), nalidixic acid (71.3%), and ciprofloxacin (61.3%). Furthrmore, these *E. coli* isolates were obtained from non-hospitalized UTI patients.

Comparing dendrogram results to biogram and biochemical profiles (API20E) of the nine tested isolates, we find closely similar reaction patterns between isolates 6 and 7 throughout, thus presenting an index of closest similarity of 22% of the total nine isolates tested by RAPD. Relatedness of other isolates vary in narrow or wide proportions when considering collective data of biogram, biochemical, and RAPD profiles. Isolates 6 and 7 may very likely share a common source of habitat of food or water, or else contaminated vehicle.

Compared to our finding, Vogel *et al.* (2001[16]) found indistinguishable patterns by RAPD analysis and ribotyping within nine *E. coli* isolates from

cerebrospinal fluid from a total of 29 assayed types (31%), however this study used only two primers.

In the past, dendrogram based analysis of the RAPD profiles of various bacteria allowed understanding of the genetic relationship between isolates grouped into several clusters. These phylogenetic studies successfully showed the predominance of a single epidemic strain that was transmitted between hosts and its persistence over a period of time (Gomes *et al*. 2005[8]**)**. In our current study, we show a delineation of *E. coli* serotypes in the dendrogram, which is suggestive of a diversity pattern among the nine *E. coli* serotypes.

Current literature does not include direct influence of G+C ratio in RAPD primer on number and weight of isolated bands. The wide notion is that usually a random selection of 10-base primers, with a G+C content of 60% to 70%, is used for amplification; and use of A+T rich primers generally does not produce amplification products (Patwary *et al*. 1993[15]). However, current literature rarely depicts RAPD analysis using high number of primers.

Primer OPO-11 has the heaviest MW of 3108 and yielded 10 different bands. The primer notably has the highest G ratio (50%) among all used primers. Nonetheless, number of different bands cannot be a consequence of molar ratio of primer because of random distances generated upon primer annealing.

Pacheco *et al*. (1996[14]) observed that oligos with high G+C content (>60%) resulted in a greater and better reproducible number of strain specific bands in enterotoxigenic *E. coli*. On the contrary, the size and G+C content of the arbitrary primers did not seem to be an essential requirement for the generation of more informative RAPD profiles in *E. coli*. Similarly

in our RAPD studies, we did not observe influence of G+C content of the primer on the number of polymorphic bands. The most challenging aspect of a protocol for bacterial subtyping by RAPD analysis was the selection of suitable primers. We found that primers OPA-14 and OPA-15 generated most numbers (13 and 11 respectively) of entirely different banding pattern for each serotype and could differentiate all the serotypes from one another. Hence, we suggest these primers for molecular typing of *E. coli* isolates as they disclosed wide inter serotype variations. Clearly primers with comparable or higher G+C content yielded less number of bands.

A phylogenetic tree was generated from the diverse *E. coli* RAPD patterns obtained in this study. The dendrogram generated from different primers was branched, suggestive of a genetically diverse population of nine uropathogenic *E. coli* causing UTI in Khoms area.

References:

1. *Abraham S, Chapman TA, Zhang R, Chin J, Mabbett AN, Totsika M, Schembri Mma. (2012). Molecular characterization of Escherichia coli strains that cause symptomatic and asymptomatic urinary tract infections. J Clin Microbiol 50(3), pp. 1027-1030.*

2. *Beatriz E, and Guth C. (1997). Random amplification of polymorphic DNA reveals serotype specific clonal clusters among Enterotoxogenic Escherichia coli strains isolated from humans. J Clin Microbiol 6(35): 1521-1525.*

3. *Cave H, Bingen E, Denamur FJ. (1994). Differentiation of Escherichia coli strains by polymorphic DNA analysis. Res Microbiol 145(2): 141-150.*

4. *Cheng, HR. and Jiang N. (2006). Extremely rapid extraction of DNA from bacteria and yeasts. Biotechnol Lett 28:55–59.*
5. *Di-Bonavenura GA, Ricci E, Loggia ND, Catamo G, and Piccolomini R. (1998). Evaluation of E-test for antimicrobial susceptibility testing of Pseudomonas aeruginosa isolates from patients with long term bladder catheterization. J Clin Microbiol 824:836.*
6. *Ejrnæs, K. (2011). Bacterial characteristics of importance for recurrent urinary tract infections caused by Escherichia coli. Dan Med Bull 58(4), B4187-109.*
7. *Garg V, Bose A, Jindal J, Goyal A. (2015). Comparison of clinical presentation and risk factors in diabetic and non-diabetic females with urinary tract infection assessed as per The European Association of Urology. J Clin Diagn Res 6:PC12-14*
8. *Gomes, A.R., L. Muniyappa, G. Krishnappa, V.V.S. Suryanarayana, S. Isloor, B. Prakash and P.G. Hugar, (2005). Genotypic characterization of avian Escherichia coli by random amplification of polymorphic DNA. Int J Poult Sci 4: 378-381.*
9. *Kumar NS, Gurusubramanian G. (2011). Random amplified polymorphic DNA (RAPD) markers and its applications. Sci Vis 11 (3), 116-124.*
10. *Lees, GF. (1996). Use and misuse of indwelling urethral catheters. Vet. Clin. North Amer. Small Anim Pract 26(3):499.*
11. *National Committee for Clinical Laboratory Standards (NCCLS). (1975). Performance standards for antimicrobial disc susceptibility*

tests. National Committee for Clinical Laboratory Standards, Villanova, PA. USA.

12. *Neamati F, Firoozeh F, Saffari M, and Zibaei M. (2015). Virulence genes and antimicrobial resistance pattern in uropathogenic Escherichia coli isolated from hospitalized patients in Kashan, Iran. Jundishapur J Microbiol 8(2): e17514.*
13. *Nielsen KL, Dynesen P, Larsen P, Frimodt-Moller N. (2014). Faecal Escherichia coli from patients with E. coli urinary tract infection and healthy controls who have never had a urinary tract infection. J Med Microbiol 63, 582-589.*
14. *Pacheco A, Guth B, DeAlmeida D, Ferreira L. (1996). Characterization of enterotoxigenic Escherichia coli by random amplification of polymorphic DNA. Res Microbiol 147: 175-182.*
15. *Patwary MU, Mackay RM, van der Meer JP. (1993). Revealing genetic markers in Gelidium vagum (Rhodophyta) through the random amplified polymorphic DNA (RAPD) technique. J Phycol 29: 216–222.*
16. *Vogel L, van Oorschot E, Maas HM, Minderhoud B, Dijkshoorn L. (2000). Epidemiologic typing of Escherichia coli using RAPD analysis, ribotyping and serotyping. Clin Microbiol Infect 6:82-87.*
17. *Welsh J, McClelland M. (1990). Fingerprinting genomes using PCR with arbitrary primers. Nucleic acids Res 18:7213-7218.*