Plant Archives Vol. 19 No. 2, 2019 pp. 2775-2780 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

CONFIRMATION OF ROUTINE IDENTIFICATION OF SOME BACTERIAL SPECIES ISOLATES FROM UTI INFECTED WOMEN USING GENE SEQUENCING AND 16 SRRNA TECHNIQUES

Shaymaa Naji Dahham[1], Nadira S. Mohamed[2], Marwa Hasan Abdullwahab[3], Asmaa Easa. Mahmood[4] and Huda dhamin Abdaljabbar[5]

[1]Department of Biology, College of Education, University of Samarra, Iraq.
[2]Forensic DNA Center for Research and Training, Al-Nahrain University, Iraq.
[3]Department of Biology, College of Science, University of Tikrit, Iraq.
[4]Department of Pathological Analyzes, College of Applied Sciences, Samarra University, Iraq.
[5]Department of Biology, College of pure sciences, University of Tikrit, Iraq.

Abstract

The study had included isolation and identification of 140 samples of women attended Tikrit Teaching Hospital infected with urinary tract their ages were between (15-45) during the period from 1/4 / 2016 to 1/4/2017. This study clarified that (101) sample from the total samples showed bacterial growth in percentage (72.1%). It was clear according to the results of diagnosis that *Escherichia coli* had the highest proportion of isolations 39.6% from the total isolates followed by both *Staphylococcus warnei* and *Klebsiella pneumoniae* 11.8%. *Enterococcus faecalis* 9.9%, *Proteus miribilis* 6.9%. *Pseudomonas aeruginosa* 5.9%, both *Streptococcus sobrinus* and *Pseudomonas stutzeri* 4.9% *Proteus vulgaris* had the least percentage 3.96% by four from (101) isolates. The sensitivity of these isolates was tested by (10) antibiotic which included quinolones, aminoglycosides, beta- lactams and tetracyclines antibiotics. DNA has been extracted for 18 bacterial isolates (triplicate for each type) 3 isolates of Gram positive (*Staphylococcus warnei, Streptococcus faecalis and streptococcus sobrinus*) and Gram negative bacteria (*E. coli, Klebsiella pneumonia and Proteus mirabilis)* were included for molecular diagnosis after routine and Vitek 2 diagnosis. Results showed a single band with molecular weight 1411bp for 16sRNA gene for different isolates. The results of PCR confirmed by gene sequencing that chosen for molecular diagnosis were characterized by compatibility of the laboratory diagnosis with in molecular diagnosis and the isolates were compatible with results recorded in WHO gene bank.

Key words: UTI, PCR, gene sequencing, 16Sr RNA

Introduction

UTI is a respond of urinary tract due to microbial attack; UTI is more abundant bacterial infection in human especially the infection of urinary bladder and penis (Sim., 2001). UTI is defined as the presence of Microorganisms especially bacterial urinary tract which considered sterile (Darko, 2012). Urinary tract infections are must abundant in female than male because of the penis in women is shorter and the place were near the anus (Tortora *et al.,* 2010). In both male and female there are a limited number of bacteria in the upper part of urinary stream (Cowan, 2009), the urinary tract has a different mechanisms of defense such as, urine flux, acidic pH medium of urine and anti-bacterial polypeptides (Sansonetti, 2010). The most affect organs in the urinary system is the urinary bladder (cystitis), the urethra (ureteritis) and the kidney (pyelonephritis) (Vandepitte *et al.,* 2003). UTI may occur with or without symptoms and may cause a series complications if it not be treated (Brown and Smith, 2015; Pezzlo, 1988). The symptoms of UTI is the presence of lower abdomen pain, uncomfortable urination, the urine appears dark or cloudy because of the presence of puss and epithelial cells and consequently fever. UTI occurs due to the respond of Urothelium to Invasion and colonization of bacteria and this always charazterized by

the presence of bacteria in the urine (Bacteriuria) and puss cells (pyuria). The infection may be occurs anywhere in UT and was accomplished with a lot number of bacteria with the presence of the signs and symptoms of infection (Tangho and McAminch, 2008), the term of UTi is defined as the presence of more than 10^5 bacterial cell/ml of urine (Brigul *et al*., 2006). UTI is a predominant disease in the whole population at any age for both sex, however females are more susceptible to UTI than male due to short women urethra and the near of urinary drain hole from anus. One of five women are infected at least one time during her entire life (sweet and Gibbsm 2009), many factors leads to UTI including; Diabetes mellitus, pregnancy, prostate enlargement and renal calculi (Lewis and Chamberlain, 1992; Linda and Frederic, 1989; Jennet *et al*., 2006). Because of the increase of UTI among women in particular and the advances in technologies in the routine identification of UTI bacteria using Vitek 2, The study aimed to isolate the most important factor of UTI in women and identified by routine methods and the confirmation of bacterial isolate using Vitek 2, molecular characterizations using 16SrRNA and confirmation of the gene compatibility with NCBI genome bank using sequencing technique.

Materials and Methods

Isolation and identification of bacterial isolates

140 urine samples were collected from women with UTI attended to Tikrit educational hospital during the period December, 2016–April, 2017. Women age were 15-45 years, they were suffering from hard of urination and burning during urination. Urine samples were collected in sterile plastic containers and patients were asked to wash the genital tract with soap to avoid contamination and collect samples of the midstream Urine (Vandepitte *et al.,* 2003). Samples were cultures on blood agar, Mannitol salt agar and MacConkey agar medium, in order to isolate the negative and positive bacterial species, dishes were incubated at 37º in aerobic environment for 24-48 hours. Colonies and isolates were the transferred to appropriate medium and the colonies were identified using biochemical methods (e.g. oxidase, catalase and Imvic, then the identification were confirmed using Vitek 2.

Preservation of the Bacterial Isolates

Bacteria was preserved for short time and longtime according to (Collee *et al.,* 1996).

Antibiotics

Antibiotic discs of Ampicillin (AM), Cefoxitin (FOX), Chloramphenicol (C), Trimethoprim (TMP), Tetracycline (TE), Imipenem (IPM), Gentamicin (GN), Tobramycin (TN), Rifampin (RA), Amikacin (AK) from Bioanalyze were used to investigate the bacterial isolates. The symbols, concentrations, inhibition areas according to Clinical and Laboratory Standards Institute (CLSI, 2014).

Molecular detection

DNA was extracted from samples using DNA extraction kit (Genomic DNA Mini Kit, USA, Catalog GB 100) according to the manufacturer's protocol.

Detection of 16sRNA gene was conducted by using primers (Forward: 52-AGA GTT TGA TCC TGG CTC AG-3, and Reverse 52 GGT TAC CTT GTT ACG ACT T-3) were supplied by IDT (Integrated DNA Technologies) company, Canada for amplification of fragment 1411bp of partial 16sRNA gene. (blaKPC2F:5'total volume of 25µl containing 1.5µl DNA, 12.5 µl Taq Master Mix PCR (Promega, USA), 1µl of each primer (10 pmol) then distilled water was added into tube to a total volume of 25µl. PCR amplification was conducted under the following conditions: 5 min at 95°C, followed by 35 cycles of 94°C for 45sec, 56°C for 80sec, and 72°C for 90 sec and a final extension of 72°C for 7 min using a thermal Cycler made by Labnet (Labnet international, Inc, MultiGeneOptiMax, Catalog #: TC9610-230, USA). The PCR products were separated on a 1.5% agarose gel electrophoresis and visualized by exposure to ultra violate light (302nm) after red safe staining, target band was re-extracted from gel by gel PCR DNA fragment extraction kit under cat. No. DF/100. Primers were supplied from Integrated DNA Technologies company, Canada (IDNAT) were used in this study, The PCR Master Mix contain 1.5 µl DNA, 5 µl of TAQ PCR PCR ORIMIX (Interon, Korea), 1 µl from each primers (10 pmol) and DW were added to a tube to get final concentration 25µl (Table 3). PCR run program as fellow Initial Denaturation 1 cycle for 5 min at 94°C, and 35 cycles of Second denaturation 30 sec at 94°C, Annealing for 40 sec at 62°C, and Extension for 1 min at 72°C; 1 cycle final Extension for 7 min at 72°C. DNA has been extracted form 18 bacterial isolates including gram positive bacteria (*Staph.warnei, Strep. faecalis and strep sobrinus*) and gram negative bacteria (*E.coli, Klebsiella pneumonia and Proteus mirabilis*) and submitted to molecular diagnosis. The purity and concentration of DNA was estimated using Nanodrop instrument, the purity of samples were (1.6-1.9) represented a good quality (Sinha *et al*., 2001). The purity was confirmed by gel electrophoresis before using on PCR (Wu *et al*., 1991).

The sequencing and Compatibility

The PCR products were submitted for sequencing

in National Instrumentation Center for Environmental Management (NICM) Company online at (http://nicem.snu.ac.kr/main/?en_skin=index.html), biotechnology lab, machine is DNA sequencer 3730XL, Applied Biosystem), compatibility search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov) and BioEdit program. The phylogenetic tree was drawn to scale using the UPGMA method including 27 nucleotide sequences, conducted in MEGA6 (Tamura *et al*., 2013) and the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004). The branch length = 2.02222119, and depending on the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 793 positions in the final dataset.

Results and Discussion

Results showed that 101/140(72.1%) samples from total UTI infected pregnant women was succeed to cultivated on the media table 1. Our results is closed to (Al-Mayahi, 2005) who found that (60.05%) urine samples were grew this findings may be related to many reasons, UTI may be occurred due to other infection causes rather than bacterial causes such as viral, fungal or anaerobic bacteria that cannot be isolated using routine culture methods and may they need special culture media, or may be due to the use of antibacterial that leads to disappearance of UTI bacteria. (Balat and Hill, 2003).

Identification of Bacteria

The bacterial isolates were diagnosed from urine samples of pregnant women according to (Collee *et al*., 1996; Macfaddin, 2000; Atlas, 2005, Mahon *et al*., 2015). *Escherichia coli, Klebsiella. pneumoniae. Pseudomonas aeruginosa Pseudomonas stutzeri, Proteus mirabilis, Proteus vulgaris, Staphylococcus warnei, Enterococcus faecalis*. Results showed that *E. coli* was the higher percentage among bacterial isolates (40 isolates, 39.6%), this result agrees with (Al-Khayat, 2008), who studied UTI in different places in Iraq they found that *E.coli* was in percent (48.9-67.7%), (Aka and Haji, 2014) found that *E.coli* presents in 195, (48.0%) in UTI infected women in Erbil city, also the result was agree with (Al-Tikriti, 2016) found that *E.coli* presents in 52 (46.42%) in UTI infected women in Erbil city, while the result was disagree with (Al-Hilali, 2005) who recorded (11%) for *E.coli* among other species, also the result disagree with (Vellinga, 2010) studied more than 20000 patients suffer from UTI in different places in Ireland, *E.coli* percentage was (14.4%), the difference occurred due to the geographical properties of the isolation place and the difference in environmental circumstances.

Table 1: Numbers and percent of negative and positive of bacterial growth for samples from infected women.

source of isolates	Positive samples		Negative samples		Total samples	
	number	%	Number	%	number	%
UTI	101	72.1	39	27.9	140	100

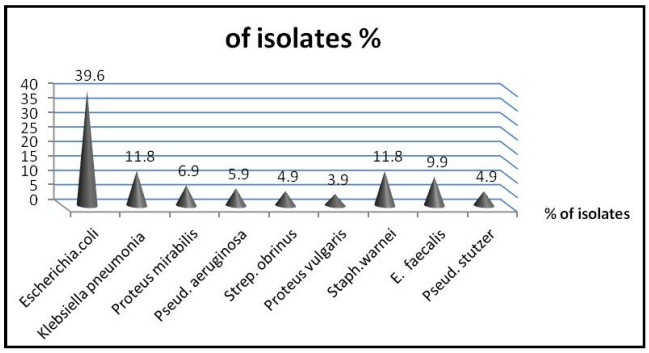

Fig. 1: Percentage of bacterial species isolated from UTI in women.

The high percent of *E.coli* isolates from UTI is due to the presence if this bacteria in the human digestive system, and can pass to the urinary tract of infected person, this bacteria is especially when the environment is adequate (Roborts, 1996). It's supposed that the cause of UTI by *E.coli* that *E.coli* presence in the feces, this make it a source of auto infection, they leave its original place (the intestine) to the UT and cause infection (Grunberg *et al*., 1996). Also it has many virulence factors such as; Fimbriae that help to adhere with Specific receptor molecules on the epithilail cells in the UTI (Braunwald *et al.,* 2001).

Table 2: Antibiotic susceptibility of bacterial isolates.

AK	RA	TN	GN	IPM	TE	TMP	C	FOX	AM	Antibiotic
										Bacteria
R	R	S	S	R	R	R	R	R	S	*E.coli*
S	R	R	R	S	R	R	S	R	R	*K. pneumonia*
S	R	S	R	R	R	R	R	R	R	*P.mirabilis*
S	R	S	R	S	R	R	R	R	R	*Pseud.*
										aeruginosa
S	S	S	R	S	S	R	S	S	S	*Strep. obrinus*
R	R	S	R	R	R	R	R	R	R	*P.vulgaris*
R	R	S	S	R	R	R	R	R	R	*Staph.warnei*
R	R	S	S	R	R	R	R	R	R	*E. faecalis*
S	R	S	R	S	R	R	R	R	R	*Pseud. stutzer*

AM: Ampicillin, FOX: Cefoxitin, C: Chloramphenicol, TMP: Trimethoprim, TE: Tetracycline, IPM: Imipenem, GN: Gentamicin, TN: Tobramycin, RA: Rifampin, AK: Amikacin.

Examination of Antibiotic sensitivity

The current study has displayed that the bacteria isolated from UTI have a high degree of resistance to most of the antibiotics under investigation. (Table 2) showed that *E.coli* isolates showed absolute sensitivity to Ampicillin, Gentamycin, and Tobramycin. This result agree with (Al-Tikriti, 2016), while the result disagree with (Al-Janabi, 2013) he reported a high resistance to all antibiotics. *Klebsiella pneumonia* showed absolute resistance to Ampicillin and Tetracycline and were sensitive to Chloramphenicol, Amikacin and Impienem. The results agree with (Al-Douri, 2013). *Proteus mirabilis* showed resistance to Ampicillin and Trimethoprim. *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* were showed absolute resistance to Ampicillin, Cefoxtin, Tetracyclin and Trimethoprim and a sensitivity to Amikacin and Impienem. This is may be due to mutations as a result of production of enzymes able to make modify the antibiotic molecule that controlled by R factor or the resistance may be result to changes in the bacterial permeability as a result of reduction of lipopolysaccharide and lipids (Brook *el al*., 2013).

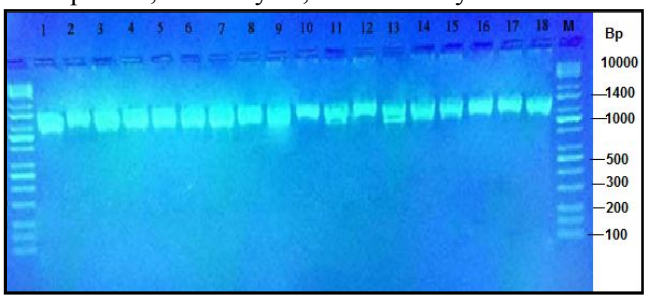

Fig. 2: Gel electrophoresis profile on 1% agarose gel stained with red safe of amplified16sRNA gene for 18 bacterial isolates from different species. Lane M represented DNA ladder (100), Amplicons band sized1411 bp.

Results showed absolute resistance of *Proteus*

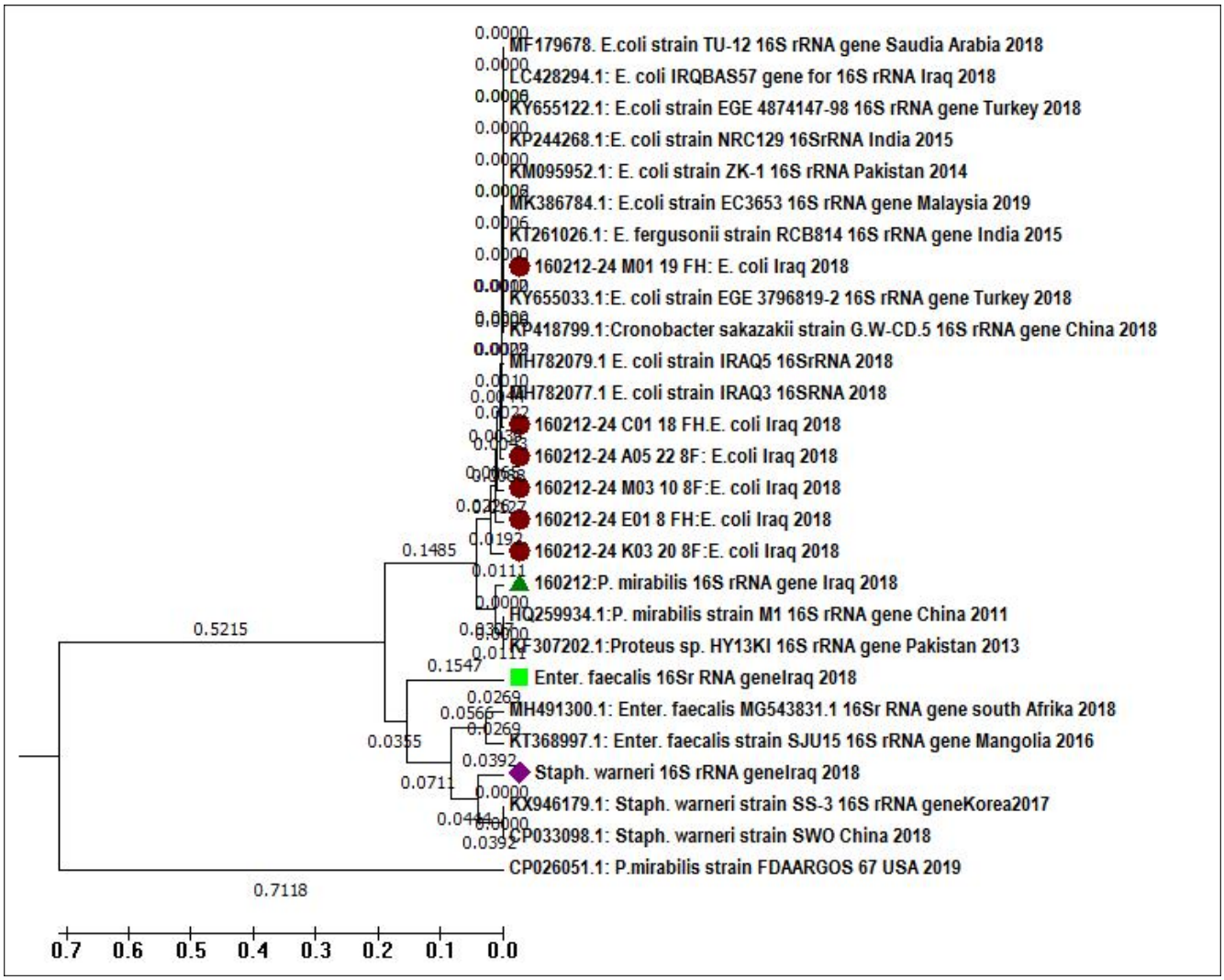

Fig. 3: The evolutionary phylogenetic relationship of 27 nucleotide sequences was conducted by UPGMA method using MEGA6. Nine of these isolates belongs to Iraqi isolates and the others imported from NCBI .The red spots represented *E.coli* strains,

vulgaris to Ampicillin and Gentamycin. While sensitive to Tobramycin. The cause of this sensitivity to this antibiotic is due to a little use of this antibiotic to pregnant women because of its effects on the embryo, therefore the bactria couldn't adapted with it, and consequently resist it, also the antibiotic is more stable against modification enzymes that modify low absorbance Aminoglycosids activity by the digestive system. *Staphylococcus warnei* showed resistance to Trimethoprim and Gentamycin but it showed sensitivity to Gentamicin. Results of sensitivity test for *Enterococcus. faecalis* were showed an absolute resistance to chloramphenicol and absolute sensitivity to Tobramycin and Amikacin. *Streptococcous sobrinus* showed absolute resistance to Trimethoprim, Gentamycin but it showed sensitivity to all other antibiotics. The high resistance to β-lactam antibiotics used in this test presented in antibiotics such as Ampicillin and Cefoxitin belongs to many mechanisms particularly the ability to produce β-lctamase enzymes that able to break beta lactam ring, change the permeability barrier or weak the affinity between the antibiotic and the target place (Cherian *et al*., 2003). The bacterial resistance to antibiotics always occurs due antibiotic long treatment by the patient or as a result of chromosomal mutations or due to movement the coding the resistance from external sources (Chambers and Deleo, 2009).

Polymerase Chain Reaction (PCR)

The molecular study has been conducted through the detection of special gene using primers specific for 16sRNA using high accurate PCR technique; the amplicons sized 1411 bp was shown in fig. 2.

The results of sequencing showed difference in molecular diagnosis with routine methods and with the diagnosis on Hicrome agar. The isolates of *E.coli* that chosen for molecular diagnosis were characterized by compatibility of the laboratory diagnosis with in molecular diagnosis and the isolates were compatible with results recorded in WHO gene bank and isolated in India by (Subbamanda *et al*., 2016) and with a study in China India by (J.W *et al.*, 2016). The 16SrRNA gene amplified with specific primers is very effective, more sensitive and rapid way for bacterial detection (Moustafa *et al.,* 2018) which is plays a critical role in the infection and treatment control.

Conclusion

While conventional microbiological methods are inadequate to identify a large diversity of bacterial species that are present in urine, genomic approaches appear to more comprehensively and quantitatively describe the urinary bacterial infection.

References

Aka, S.T. and S.H. Haji (2015). Evaluation of multi drug resistance among extended spectrum β-lactamase-producing Escherichia coli causing urinary tract infection in Erbil City. *Zanco J. Med. Sci.*, **19(2):** 998-1004.

Al-Douri, Loay Manna Ibrahim Toa'ma (2013). Determination of genetic variability of some types of bacteria isolated from UTIs in children with thalassemia using PCR technique in Tikrit City. M.Sc. thesis. College of Education. University of Tikrit.

Al-Hilali, Farah Hashim Hussein (2005). The relationship between the infection of the tonsils with *Streptococcus pyogenes* and UTI in Nineveh Governorate. M.Sc. thesis. College of Science. University of Mosul.

Al-Janabi, Amir Naji Obaid (2013). Investigation of the different pathogenic strains of *Escherichia coli* Bacteria isolated from some clinical cases of children in Babil Governorate using genetic markers. M.Sc. thesis, College of Science, University of Tikrit.

Al-Khayat, Mohammed Zaghloul Saeed (2008). Neutralization of plasmid content in bacteria isolated from patients with urinary tract infection in Mosul City. M.Sc. thesis. College of Education, University of Mosul.

Al-Mayahi, Soad Abdel-Fattah (2005). Isolation and identification of aerobic bacteria causing urinary tract infection in pregnant women in the city of Diwaniyah and its sensitivity to some plant extracts. M.Sc. thesis. College of Science. University of Basrah.

Al-Tikrity, Ibrahim Abdel Rahman Latif (2016). Detection of some virulence factors of E. coli genes isolated from patients with urinary tract infection in Erbil city. M.Sc. Thesis. College of Education, University of Tikrit.

Atlas, R.M. (2005). Handbook of Media for Environmental Microbiology. 2ed. Taylor and francis.

Balat, A. and L. Hill (2003). Genitourinary abnormalities is children with urinary tract infection., *J. Med. Sciences*, **29:** 59-63.

Braunwald, F., A. Fauci, E. kasper, C. Hauser, W. Longo and B. Jameson (2001). Harrisons principles of Internal medicine 2001, 15th edition , volum, 3, London., pp: 1620–1626.

Brigul, K., C. Ozenc, A. Altan and B. Aydan (2006). Evaluation of rapid urine screening tests to detect asymptomatic bacteriuria in pregnancy. *J. Infect. Dis.*, **59:** 261-263.

Brown, A. and H. Smith (2015). Benson's Microbiological Applications Laboratory Manual in General Microbiology. 30th edition. McGraw Hill Companies. New York.

Brooks, F.G., A.S. Morse, C.K. Carroll, A.T. Mietzner and S.J. Butel (2013). Jawetz, Melnick and Adelberg s Medical Microbiology. 26 edition. McGraw Hill Companies. USA.

Chambers H.F. and F.R. DeLeo (2009). Waves of resistance:

Staphylococcus aureus in the antibiotic era. *Nature Reviews Microbiology*, **7(9):** 629–641.

Cherian, R.P., M. Manjunath, L. Pinto and P. Prabhakar (2003). Extended spectrum β-lactamase producyion Enterobacteriaceae in a tertiary care hospital in Trinidad and Tobago. *West Indian Med. J. Mar.*, **52(1):** 3-31.

Clinical and Laboratory Standards Institute (CLSI) (2014). Performance standards for antimicrobial susceptibility testing, 24th Informational Supplement, **Vol. 34 No. 1**, Pennsylvania USA.

Collee, J.G., A.G. Fraser, B.P. Marmion and A. Simons (1996). Mackie and McCartney Practical Medical Microbiology14th ed., Longman Singapore Publishers Ltd., Singpore.

Cowan, K.M. (2009). Microbiology A systems Approach. 3ed edition. McGraw Hill Companies. Inc. USA.

Darko, S.N. (2012). Compartive Diagnosis of E.COLI-induced UTI using Dipstick, Microbiological Culturing and PCR Methods in School-Going Adolescents. M.Sc. Thesis. College of Science. Kwame Nkrumah university.

Jennett, J.C., R.H. Heptinstall, J.L. Olson, M.M. Schwartz and F.G. Silva (2006). Heptinstall's pathology of kidney. 6th ed. Wolters kluwer/Lippincott. Wiliams and Wilkins. Philadelphia, USA: 767.

J, W. (2016). Screening of Acid Producing Bacteria from Blueberry Rhizosphere. Shandong Agricultural University, College of Life Science, Daizong Street of Taishan District, Taian, Shandong 271018, China.

Lewis, T.L.T. and G.V.P. Chamberlain (1992). Obstetrics. 5th ed. Butler and Tanner, London.

Linda, T. and B. Frederick (1989). Cervicovaginal antibody in the pathogenesis of recurrent urinary tract infection in women. *J. Inf. Immun.*, 1287-1288.

MacFaddin, J.F. (2000). Biochemical tests for identification of medical bacteria, 3rd ed. Lippincott Williams & Wilkins. Philadelphia. USA.

Mahon, C., G. Manuselis and D. Lehman (2015). "Textbook of Diagnostic Microbiology", Elsevier 5rd ed. USA: Saunders Company, (868).

Moustafa, A., Li. Weizhong, Harinder Singh, Kelvin J. Moncera Manolito G. Torralba, Yanbao Yu, Oriol Manue, William Biggs, J. Craig Venter, Karen E. Nelson, Rembert Pieper, Amalio Telenti (2018). Microbial metagenome of urinary tract infection. *SCientifiC REPOrTS.*, **8:** 4333.

Pezzlo, M. (1988). Detection of Urinary Tract Infections by Rapid Methods. *Clinical Microbiology Reviews.*, **1(2):** 268-280.

Roberts, J.A. (1996). Factores predisposing to urinary tract infection in children. *Pediatr. Nephrol.*, **10:** 517–522.

Sansonetti, P. (2010). Bacterial Virulence Basic Principle, Model and Global Approaches. WILEY BLACKWELL. Germany.

Sim, J. (2001). Urinary Tract Infection. Nidus Info. Services, Inc. New York, U.S.A.

Sinha, R.P., M. Dautz and D.P. Hader (2001). A simple and efficient method for quantitative analysis of Thymine Dimers in Cyanobacteria, Phytoplankton and Macroalgae. *Acta Protozoologica*, **40:** 187-195.

Subbamanda, Y., T. Naik, M.S. Dharne and S.G. Dastager (2016). NCIM Authentication and Upgradation Project CSC0407. NCIM Resource Centre, CSIR-National Chemical Laboratory, Dr.Homi Bhabha Road, Pune, Maharashtra 411008, India.

Sweet, R.L. and R.S. Gibbs (2009). Infectious disease of the female genital tract. 5th ed. Wolters Kluwer/Lippincott. Wiliams and Wilkins. Philadelphia. USA. PP: 249-256.

Tamura, K., M. Nei and S. Kumar (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* (USA) **101:** 11030-11035.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, **30:** 2725-2729.

Tangho, E.A. and J.W. McAminch (2008). Smith's general urology. 17th ed. McGraw-Hill companies, Inc. USA. PP: 193-196.

Tortora J.G., R.B. Funke and L.C. Case (2010). Microbiology An Introduction. 10th edition. Benjamin Cummings. USA.

Vandepitte, J., J. Verhaegen, K. Engbaek, P. Rohner, P. Piot and C.C. Heuck (2003). Basic laboratory Prodceduresin Clinical bacteriology. 2nd ed. WHO. Geneva.

Vellinga, E.C., M. Contu and A. Vizzini (2010). Leucoagaricus decipiens and La. erythrophaeus, a new species pair in sect. Piloselli. *Mycologia*, **102(2):** 447-454.

Wu, D.Y., L. Vgozzoli, B.K. Pal, J. Qian and R.B. Wallace (1991). The effects of temperature and oligonucleotide primer length on the specifity and efficiency of amplification by polymerase chain reaction. *DNA and Cell Boil.*, **10:** 233-238.