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

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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 mirabilis 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° 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 Gibbison 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 37° 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 GGT 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), 1 µl 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, Strept. 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.

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

<table>
<thead>
<tr>
<th>Source of isolates</th>
<th>Positive samples</th>
<th>Negative samples</th>
<th>Total samples</th>
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<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
<td>Number</td>
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<tr>
<td>UTI</td>
<td>101</td>
<td>72.1</td>
<td>39</td>
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</tbody>
</table>

**Table 2:** Antibiotic susceptibility of bacterial isolates.

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<thead>
<tr>
<th></th>
<th>AK</th>
<th>RA</th>
<th>TN</th>
<th>GN</th>
<th>IPM</th>
<th>TE</th>
<th>TMP</th>
<th>FOX</th>
<th>AM</th>
<th>Antibiotic</th>
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<td>S</td>
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<td>R</td>
<td>R</td>
<td><em>E. coli</em></td>
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<td><em>K. pneumonia</em></td>
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<td><em>P. mirabilis</em></td>
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<td>R</td>
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<td><em>P. aeruginosa</em></td>
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<td><em>Strep. obirius</em></td>
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<td>R</td>
<td>R</td>
<td><em>P. vulgaris</em></td>
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<td>R</td>
<td>R</td>
<td>R</td>
<td><em>Staph. warnei</em></td>
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<td><em>P. stutzer</em></td>
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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, Cefoxin, Tetracycline 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 et al., 2013).

Results showed absolute resistance of Proteus
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 bacteria couldn’t adapt 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 warneri* 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. *Streptococcus 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-lactamase 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 16SrRNA 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**


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