THE INTERNATIONAL JOURNAL OF
SCIENCE & TECHNOLEDGE

PCR Identification of Urinary *Pseudomonas aeruginosa* from Pregnant Women Attending a Tertiary Health Care Centre in Makurdi, Central Nigeria

Aernan, P. T.
Lecturer, Microbiology Unit, Department of Biological Sciences, Federal University of Agriculture, Makurdi, Benue State, Nigeria

Abstract:
In this study 28 isolates of P. aeruginosa were obtained from the urine of pregnant women attending antenatal in a tertiary hospital in Makurdi, Central Nigeria. Urinary tract infection is one of the most frequently seen 'medical' complications in pregnancy.P. aeruginosa was isolated from (10.1) of urine samples. But using PCR- based amplification of the specific P. aeruginosaprimers proved a 100% specificity.Several results indicated that preliminary phenotypic testing had misidentified several isolates. The 16S rDNA sequence was determined for 28 isolates, and in all cases it confirmed the results of the PCR assays.

Keywords: *PCR, Pseudomonas aeruginosa, Makurdi, Central Nigeria*

1. Introduction

Common uropathogens causing complicated Urinary tract infections(UTIs) among patients include *Pseudomonas* aeruginosa and also during pregnancy decreased concentration of urine, glucosuria, and progesterone effects (promote ureteric dilatation) also influence infection (Ipe*et al.,* 2013).
The genus *Pseudomonas* is made up of Gram-negative, rod-shaped bacteria that inhabit many niches (Narins 2003; Lanini*et al.,* 2011). *Pseudomonas* species are common inhabitants of the soil, water, and vegetation. The genus is particularly noteworthy because of the tendency of several species to cause infections in people who are already ill, or whose immune systems are not operating properly. Such infections are termed opportunistic infections (Narins 2003; Nikbin*et al.,* 2012). *Pseudomonas* rarely causes infections in those whose immune systems are fully functional. The disease-causing members of the genus are therefore prevalent where illness abounds. *Pseudomonas species* are one of the major causes of nosocomial (hospital acquired) infections (Wolska and Szwada 2008; Narins 2003; Lanini*et al.,* 2011). The species that comprise the genus *Pseudomonas* are part of the wider family of bacteria that are classified as *Pseudomonadaceae*.
Pregnant women are more susceptible to urinary tract infection (UTI), owing to altered anatomical and physiological state during pregnancy (Tadesse*et al.,* 2014). Although the incidence of bacteriuria in pregnant women is similar to that in non-pregnant women, the incidence of acute pyelonephritis in pregnant women with bacteriuria is significantly increased. Pregnancy is a unique state with anatomic and physiologic urinary tract changes. While ASB in non-pregnant women is generally benign, pregnant women with bacteriuria have an increased susceptibility to pyelonephritis (inflammation of the tissues of the kidney) and low birth weight (birth weight of less than 2500 g) (Schnarr and Smaill 2008.), hence special attention to the pregnant women is one of the most important points in health care (Widmer*et al.,* 2011).
Conventional microbiological protocols for the identification of *P. aeruginosa* from both clinical and environmental samples are reliable, though they may require several days to be completed. Rapid detection of isolates causing infections is very important for consequent treatment decision of patients. PCR has the potential for identifying microbial species rapidly by amplification of sequences unique to a particular organism (Khan and Cerniglia, 1994).
Several of the early methods used to investigate the epidemiology of P. aeruginosa and to determine clonality, including serotyping, pyocin production, bacteriophage susceptibility, biotyping, and antimicrobial susceptibility were based on phenotypic differences. Some of these phenotypes lack reproducibility because expression of phenotypes of interest may change during an outbreak or under laboratory conditions. Such limitations make phenotypic methods of limited use in subtyping bacteria (Bingen et al. 1996). PCR-based techniques are yet to replace standard bacterial culture due to their complexity, cost and need for specially trained personnel. PCR-free assays, in which the genetic content of the sample could be directly analyzed, could offer a simple yet specific diagnostic tool, while alleviating or eliminating many of the constraints associated with genetic amplification. A novel assay for UTI detection can be presented. (Millar *et al*, 2007).

2. Materials and Methods

2.1. Sample Collection

The study was carried out in a tertiary hospital in Makurdi, Middle belt region of Nigeria between September 2014 and April 2015. The hospital serves as a referral Centre for over half a million people within 40 km radius of the city. The town is divided by the River Benue into the north and south banks. Owing to its location in the Benue River, Makurdi experiences warm temperature most of the year.

A purposive selection consisting of 438 pregnant women attending the ante-natal clinic was taken. This included women in the three trimesters of pregnancy. Patients were excluded if they had symptoms of urinary tract infection, had taken antibiotics during the previous week, or had any signs of labor.

Written informed consent was obtained from the women for the collection of each specimen, in accordance with the ethical guidelines of the medical Institution.

Each of the women were instructed on how to collect a clean-catch midstream urine sample in a sterile container

2.2. Processing and Isolation of Samples

The culture media used for isolation were Cystein-Lactose Electrolyte-Deficient (Difco Co, USA), Blood and chocolate agar plates. Each urine sample was inoculated and streaked with·; the aid of heat-flamed standard wire loop (delivering 0.001 ml urine) on to the agar plates. The plates were incubated aerobically at 37°C for 24hrs and then examined. Only plates with significant growth (i.e. at least 100cfu/ml) were considered significant and further analyzed. The cultural and morphological characteristics of distinct and isolated colonies were studied. This included size, elevation, opacity and colour. Distinct and isolated colonies from each significant growth were Gram stained. Those resembling Pseudomonas were inoculated onto Cystine Lactose Electrolyte Deficiency (CLED) and colonies that did not ferment Lactose were presumptively identified as *Pseudomonas aeruginosa* and confirmed by the oxidase slide and tube agglutination tests. *Pseudomonas aeruginosa* are usually Oxidase positive. Plates were then viewed under UV rays to observe a characteristic fluorescent. Gram-negative rods were identified as lactose or non-lactose fermenters using Eosin Methylene Blue (EMB and MacConkey agar. Plates were further sub cultured on a selective medium using *Pseudomonas* Chromagar and Centimide agar, this was incubated for 24hrs at 37°C. Plates were observed for green-mauve color on Pseudomonas chromagar and yellow green or yellow brown on centrimide agar, again plates were viewed under UV rays for fluorescent. Suspected organisms were inoculated on nutrient agar and incubated for 24hrs at 37°C. Colonies from the nutrient agar were presented for the Gram stain, Antibiotic Sensitivity tests, Biochemical tests which include; Citrate, Indole, Urease, Oxidase tests were carried out. Each isolate originating from a single colony of each patient's culture was identified as *P. aeruginosa* by analytical profile index test kit (API 20 E test; bioMérieux.).

2.3. DNA Extraction using Relia Prep Spin Column Protocol

This method is used for the purification of genomic DNA from cells by combination of silica membrane technology and micro-centrifugation. Relia prep spin Column method does not use ethanol in its purification protocol to avoid downstream problems associated by ethanol carry over. DNA samples were thus eluted with nuclease free water. The *Pseudomonas aeruginosa* cell colony were first emulsified in 200µl TE buffer, this was thoroughly mixed for at least 10 minutes at room temperature.1.5ml of the cell sample was pipetted into micro-centrifuge tubes, capped and centrifuged at 1400 rpm for at least 10s. The supernatant was discarded and the cell pellets re-dissolved in 200µl of cell lysis Buffer CLDwith the addition of 25µl Proteinase K, mixed thoroughly by vortexing. The tubes were incubated at 56^{0} C for 2hrs to allow lysing of the cells. A relia prep Binding Column was placed into an empty collection tube. This mixture was transferred into the relia prep binding columns placed in 2ml collection tubes and centrifuged at 1400 rpm for 1min. The flow through and the collection tubes were discarded. The columns were placed in new set of collection tubes and 500µl Buffer CWD1 was added into the column to wash the sediments, centrifuged at 8000rpm for 1min. The flow through and collection tubes were again discarded. The columns were placed in new set of collection tubes and 500µl Buffer CWD2 was added into the column for a second wash, centrifuged at 14000 rpm for 3mins. This step was repeated by adding 500µl Buffer CWD3 for a third wash, centrifuged at 1400rpm for 3mins. The column was thereafter placed in a clean 1.5ml micro-centrifuge tube and 50µl of nuclease-free water added to the column. This was centrifuge for 1 min at maximum speed (14000) rpm to elute the DNA. The Relia Prep Binding Column was discarded and the flow through containing the DNA was collected and used to run the gel electrophoresis and Polymerase Chain Reaction (PCR).

2.4. Primer Selection for Pseudomonas Aeruginosa

The *algD* GDP mannose dehydrogenase gene of *P. aeruginosa* contains 2032 bp (GenBank, access no. 400337, identification no. g45267) and its expression results in a 48-kDa protein, the enzyme GDP mannose dehydrogenase, the first enzyme that is specific for alginate production in the alginate biosynthetic pathway (Dereтic*et al.,* 1987). The promoter region of this gene is well known for its regulatory role in the transcription of other genes involved in alginate biosynthesis (Govan and Deretic, 1996). The selected primers VIC1 (5′ TTC CCT CGC AGA GAA AAC ATC 3′) and VIC2 (5′ CCT GGT TGA TCA GGT CGA TCT 3′) were designed to amplify a 520-bp segment of the *algD* GDP mannose dehydrogenase gene of *P. aeruginosa*. Bacterial universal primers 11E-13B, targeting the 16S rRNA gene of bacteria (Relman, 1993) was employed to assure DNA quality.

Primer IDs	Sequence	Amplicon Size (bp)
337F-16s rRNA (Forward)	GAC TCC TAC GGG AGG CAG CAG	
518R-16s rRNA (Reverse)	GTA TTA CCG CGG CTG CGT CTT	181
VIC1-F (Forward)	TTC CCT CGC AGA GAA AAC ATC	
VIC2-R (Reverse)	CCT GGT TGA TCA GGT CGA TCT	520

Table 1: Primers used for Universal Bacterial and Primer Specific for Pseudomonas aeruginosa

3. Results

A total of 438 pregnant women urine samples was examined using culture techniques for bacteriuria. Out of which 277 were positive for significant bacteriuria (10^5cfu/ml) giving a prevalence rate of 63. 3%. Results showed that organisms present in the urine essentially belong to nine (9) genuses, predominantly Gram-negative organisms constituting 66.6% in which Pseudomonas aeruginosa constitutes 28(10.1%) See table 2. The Analytical profile index (API) test kit was used to confirm identification of 28 *P. aeruginosa* isolates.

Gram reaction	Organisms Isolated	Number (%)
Gram negative	*Pseudomonas aeruginosa*	28 (10.1)
Gram negative	*Escherichia Coli*	87(31.4)
Gram negative	*Klebsiella*spp	31(11.2)
Gram negative	*Proteus* spp	34(12.3)
Gram negative	*Serratia*spp	2(0.7)
Gram positive	*Staphylococcus* spp	60(21.7)
Gram positive	*Streptococcus* spp	10(3.6)
Gram positive	*Corynebacteria*spp	3(1.1)
Gram negative	*Enterobacter* spp	22(7.9)

Table 2: Distribution of uropathogens from pregnant women in Makurdi

After the extraction of DNA from the 28 *Pseudomonas aeruginosa* isolates, the resultants products were analyzed on gel electrophoresis preceded by a PCR run using universal bacterial primers. All the 28 *Pseudomonas aeruginosa* isolates showed genomic DNA bands. The intensity of the bands is indicative of the quality of DNA extracted. The amplicons for the positive samples were all visible at the 200 bp DNA size. See figures 1 and 2. Lanes 1 to 16 represent the first batch run of DNA Products on gel electrophoresis. L is 100 bp ladder size. Figure 2 represents the second batch run of DNA products of gel electrophoresis.

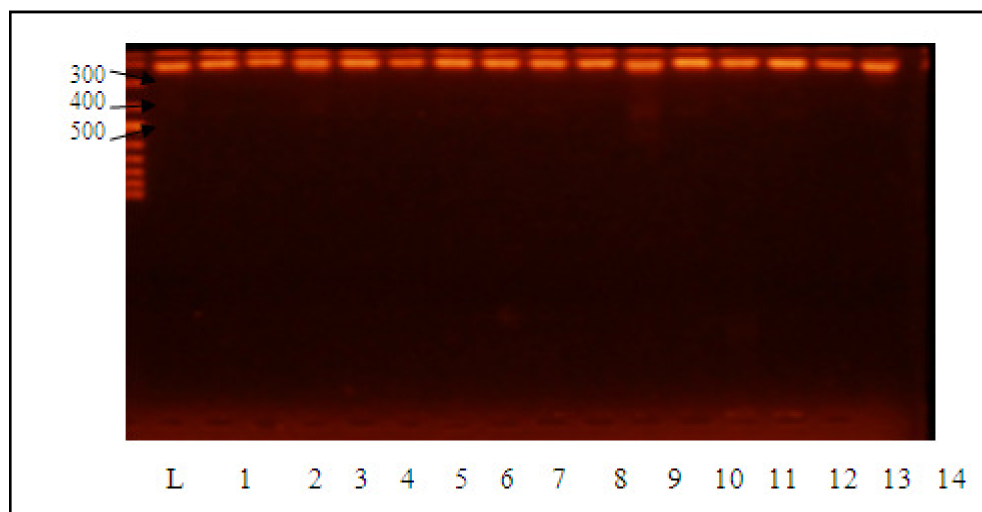

Figure 1: First batch gel electrophoresis of PCR products using universal bacteria primer. Lane 1-16 shows the 181 bp amplicon of Pseudomonas aeruginosaisolates

→ L is 100bp DNA ladder.

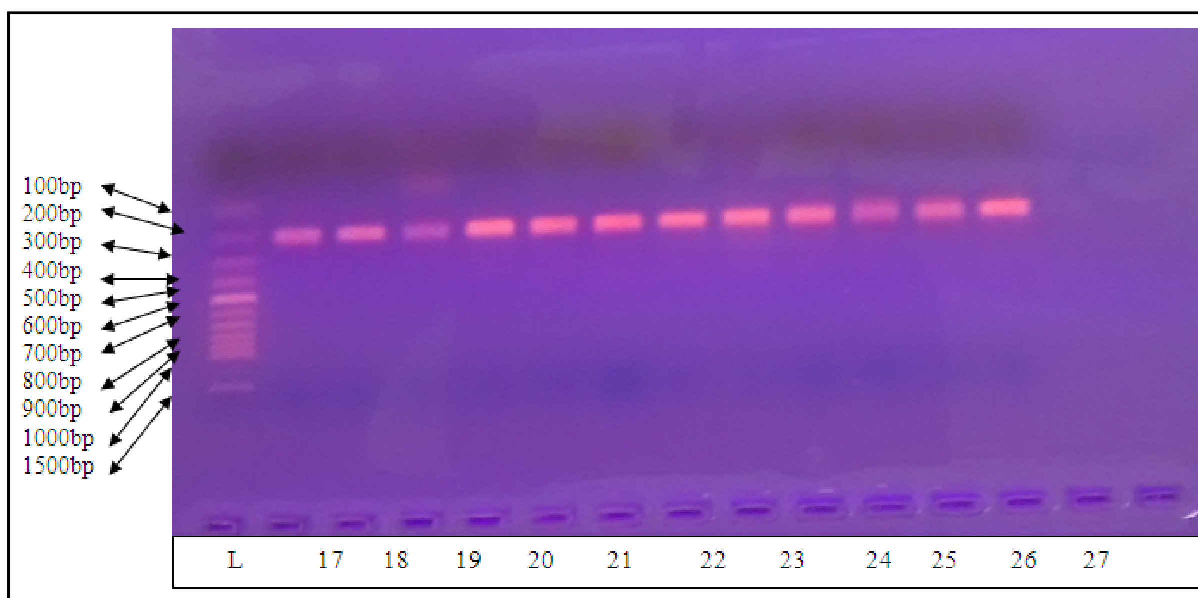

Figure 2: Second batch gel electrophoresis of PCR products using universal bacteria primer. Lane 17-28 shows the 181 bp amplicon of Pseudomonas aeruginosaisolates

→ L is 100bp DNA ladder.

Pseudomonas aeruginosa specie- specific primer was used to amplify 16S DNA segments of each isolate. The gel electrophoresis PCR products showing DNA amplicons with size 520bp is shown in figure 3.

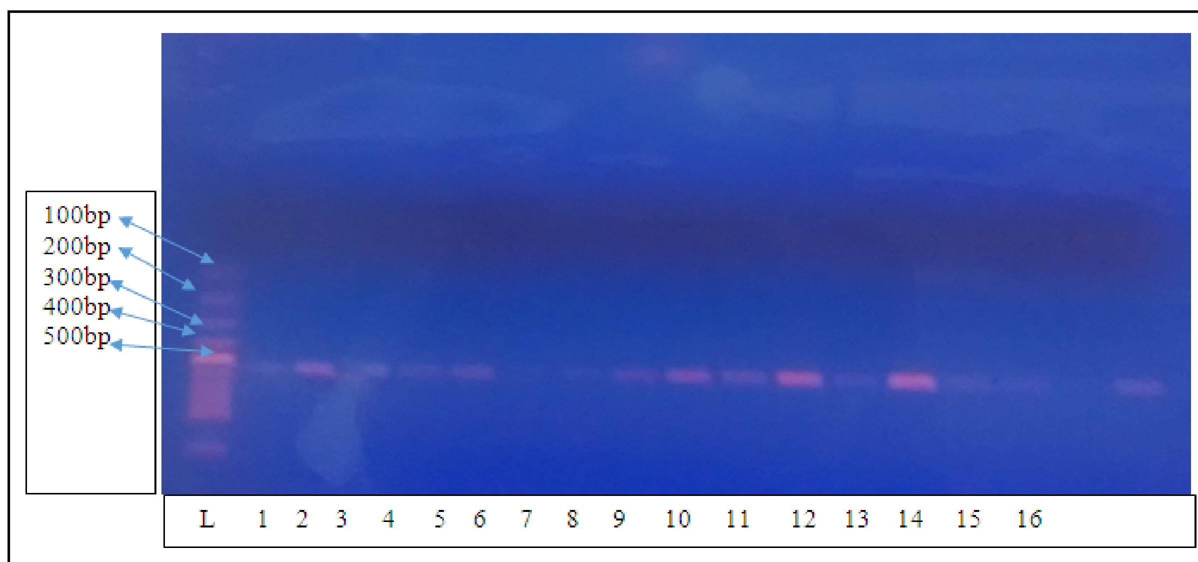

Figure 3: The gel electrophoresis PCR products using specie specific primers (520bp) for the identification of Pseudomonas aeruginosa isolates. Lane L is the 100bp DNA ladder, and lanes 15-28 are the P. aeruginosa amplicons

4. Discussion

It is now well established that detection of bacteriuria during pregnancy prevents acute pyelonephritis and decreases the rate of premature births. (Romero et al., 1989). The presence and detection of *Pseudomonas aeruginosa* in the urinary tract of pregnant women agrees with the findings ofWolska and Szweda. 2009.

Bacterial ascent is aided by conditions such as pregnancy and ureteral obstruction as these conditions inhibit ureteral peristalsis. Bacteria that reach the renal pelvis can penetrate the renal parenchyma through the collecting ducts and disrupt the renal tubules. In healthy individual's infection of the kidney through the haematogenous route is uncommon. On rare occasions bacteria from adjacent organs may penetrate the urinary tract via the lymphatics. Conditions associated with the lymphatic route are retroperitoneal abscesses and severe bowel infections.

Urine itself is normally sterile, but the moist environment of the periurethral area, the proximity of the urethral orifice to the rectum, and the short length of the urethra provide a conducive environment for the growth and ascension of potential uropathogenic microorganisms into the urinary system.

Common uropathogens causing complicated UTIs among patients include *Pseudomonas aeruginosa*andthe production of extra cellular proteases adds to the organism's virulence by assisting in bacterial adherence and invasion (Ipe*et al*., 2003).
P. aeruginosa was isolated from 28 (10.1) of urine samples. But using PCR- based amplification of the specific *P. aeruginosa*primers proved a 100% specificity which is comparable to results of other studies. (Blanc *et al.,* 2004; Orsi et al., 1994).
In this study, all 100% of the isolates harbored DNA and no assessment of plasmids was made. Some authors assumed that plasmid profiling cannot be used as an epidemiological marker of P. aeruginosa strains in the hospital environment and that the study of the more stable and constitutive chromosomal DNA may be more predictive for clonality (Shahcheraghi*et al*., 2003). However, in a setting with low resources, where the expensive instruments and reagents are not available, plasmid profiling can be a simple tool for epidemiologic studies.
According to some studies, detection of *P. aeruginosa* by PCR of oprI and oprL genes has a high sensitivity but a low specificity. The reason of low specificity of oprI and oprL genes is that, although the entire genome of *P. aeruginosa* has been sequenced, the genomes of its closest relatives have not. Thus, presence of false positive results among other species of bacteria during PCR assay of oprI and oprL genes indicate that they may have some similar sequences to oprI and oprL genes in their genomes (Qin *et al*.,2003). Consequently, use of only single gene target for molecular identification of *P. aeruginosa* potentially suffers from the same polymorphisms that complicate biochemical identification of this organism.

5. Conclusion

This study determined the presence of *Pseudomonas aruginosa* in urinary tract of pregnant women using PCR methods. DNA sequencing techniques allow for more specific assessment of *Pseudomonas aeruginosa*organism than do current clinical diagnostic standard, offering potential for significant clinical advancement of diagnostic methods used in routine urinary tract infection screening. The presence of this organism can pose a threat for mother and baby consequent of pyeloneohritis.

6. References

i. Bingen, E., S. Bonacorsi, P. Rohrlich, M. Duval, S. Lhopital, N. Brahimi, E. Vilmer, and R. Goering. (1996). Molecular Epidemiology Provides Evidence of Genotypic Heterogeneity of Multidrug-Resistant Pseudomonas aeruginosa Serotype 0:12 Outbreak Isolates from a Pediatric Hospital. Journal of Clinical Microbiology, 34(12):3226–3229.
ii. Blanc DS, Nahimana I, Petignat C, Wenger A, Bille J, Francioli P. (2004):Faucets as a reservoir of endemic Pseudomonas aeruginosa colonization/ infections in intensive care units. Intensive Care Med.,301964-8.
iii. Ipe, D.S., Sundac, L., Benjamin Jr, W.H., Moore, K.H. and Ulett, G.C. (2013). Asymptomatic bacteriuria: prevalence rates of causal microorganisms, etiology of infection in different patient populations, and recent advances in molecular detection. Federation of European Microbiological Societies, Minireview from Microbiological Letters 346; 1–10
iv. Khan AA, Cerniglia CE, (1994). Detection of Pseudomonas aeruginosa from clinical and environmental samples by amplification of the exotoxin A gene using PCR. Appl Environ Microbiol 1994; 60: 3739-3745.
v. Lanini, S.,SilviaD'Arezzo, S., Puro, V., Martini1, L., Imperi, F., Piselli, P., Montanaro, M.,Paoletti, S., Visca, P. and Ippolito, G. (2011). Molecular Epidemiology of a Pseudomonas aeruginosa Hospital Outbreak Driven by a Contaminated Disinfectant Soap Dispenser. Plusone, 6(2): 1-10
vi. Millar BC, Xu JR, Moore JE. (2007). Current Issues in Molecular Biology; 9:21–39. [PubMed: 17263144]
vii. Narins, B. (2003). World of Microbiology and Immunology. Volume 1 and 2. Gale Group Inc., Michigan, 465-466.
viii. Nikbin, V.S., Aslani, M.M., Sharafi, Z., Hashemipour, M., Shahcheraghi F. and Ebrahimipour, G.H. (2012). Molecular identification and detection of virulence genes among Pseudomonas aeruginosa isolated from different infectious origins. Iranian Journal of Microbiology, 4(3): 118-123.
ix. Orsi GB, Mansi A, Tomao P, Chiarini F, Visca P.(1994): Lack of association between clinicaland environmental isolates of Pseudomonas aeruginosa in hospital wards. J Hosp Infect., 27:49-60
x. Romero R, Oyarzun E, Mazor M, et al.(1989). Meta-analysis of the relationship between asymptomatic bacteriuria and preterm delivery/low birth weight. ObstetGynecol 73:576–582.
xi. Shahcheraghi, F. ;Feizabadi, M.M.; Yamin, V.; Abiri, R. and Abedian Z. (2003): Serovar determination, drug resistance patterns and plasmid profiles of Pseudomonas aeruginosa isolated from burn patients at two hospitals of Tehran (IRAN). Burns; (29): 547–551
xii. Schnarr, J. and Smaill, F. (2008). Asymptomatic bacteriuria and symptomatic urinary tract infections in pregnancy. European Journal of Clinical Investigation, 38 (S2): 50–57
xiii. Tadesse, E., Teshome, M., Merid, Y., Kibret, B. and Shimelis, T. (2014). Asymptomatic urinary tract infection among pregnant women attending the antenatal clinic of Hawassa Referral Hospital, Southern Ethiopia. BioMed Central Limited Research Notes, 7:155.
xiv. Widmer, M., Gülmezoglu, A.M., Mignini, L. and Roganti, A. (2011). Duration of treatmentfor asymptomatic bacteriuria during pregnancy (Review). The Cochrane Collaboration, 12: 1-34.
xv. Wolska, K. and Szweda, P. (2009). Genetic features of clinical Pseudomonas aeruginosa strains. Polish Journal of Microbiology, 58(3): 255–260.
xvi. Qin X, Emerson J, Stapp J, Stapp L, Abe P, Burns L, (2003). Use of real-time PCR with multiple targets to identify Pseudomonas aeruginosa and other nonfermenting gram-negative bacilli from patients with cystic fibrosis. J ClinMicrobiol; 4: 4312-4317.