Detection of virulence genes in urinary *Pseudomonas aeruginosa* from pregnant women attending Antenatal clinic in Makurdi, Central Nigeria

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Abstract: Pseudomonas aeruginosa is known to possess a vast array of virulence factors that may be responsible for its pathogenicity. *P. aeruginosa* also has a large number of virulence factors including phospholipase C, both of the hemolytic and non- hemolytic type encoded by *plc H* and *plc N* gene respectively. Exotoxin A, encoded by the *tox A* gene, exoenzyme S, encoded by the *exo S* gene, Las B elastase encoded by the *las B* gene and Alginate encoded by the *alg D* gene. The aim of this study was to determine the prevalence of these six virulence genes; (*plc H, plc N, tox A, exo S, las B, and alg D*) by Polymerase Chain Reaction (PCR). In this study 28 isolates of *P. aeruginosa* were isolated from the urine of pregnant women attending antenatal in a tertiary hospital in Makurdi, Central Nigeria.The amplification of the total DNA extracts using singleplex polymerase chain reaction detected *alg* D in 2 (7.14%), *Las B* in 3 (10.7%), *tox A* in 9(32.2%), *plc H* in 1(3.6%) and *plc N* in 5(17.9%) of the samples. The *exo S* gene was not detected in the genome of the *Pseudomonas aeruginosa* bacteria isolates. Determination of different virulence genes of *P. aeruginosa* isolates suggests that they are associated with different levels of intrinsic virulence and pathogenicity.The presence of virulent strains of *P. aeruginosa* within the urinary tract of pregnant women calls for concern as this can contribute to development of complications associated with pyelonephritis, which may in turn lead to adverse outcomes for the baby and the mother, such as premature delivery, low birth weight infants, preeclampsia, hypertension, renal failure and fetal death.

Index Terms: **Virulence, *Pseudomonas aeruginosa,*** pregnant women**, Central Nigeria.**

I. INTRODUCTION

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 asymptomatic bacteriuria (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).
Common uropathogens causing complicated 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).
Pseudomonas aeruginosa is a Gram-negative bacillus, non-sporing, non-capsulate, that occurs as single bacteria, in pairs and occasionally in short chains. *Pseudomonas aeruginosa* is an obligate aerobe that grows readily on many types of culture media, sometimes producing a sweet or grape-like odour. Some strains hemolyze blood. It forms smooth round colonies with a fluorescent greenish colour that can often produces the non-fluorescent bluish pigment pyocyanin, which diffuses into the agar. *Pseudomonas aeruginosa* in a culture can produce multiple colony types giving the impression of a culture of mixed species of bacteria. Cultures from patients with cystic fibrosis often yield *P. aeruginosa* organisms that form very mucoid colonies. It is oxidase positive, does not ferment carbohydrate, but many strains oxidize glucose. Identification is usually based on colonial morphology. It is a frequent cause of nosocomial infections, urinary tract infection and bacteraemia. *Pseudomonal* infections are complicated and can be an opportunistic pathogen. It rarely causes disease in healthy persons. The pathogenesis of *pseudomonas aeruginosa* infections is multi factorial and complex. (Narins, 2003).
Pseudomonas aeruginosa produces various virulent factors to colonize the cells of its host. Many of these factors are controlled by regulatory systems involving cell-to-cell signaling (Van Delden and Iglewski, 1999). Among these are; exotoxin A, exoenzyme S, las B elastase, phospholipases C and alginate. Endotoxin A encoded by the *tox* A gene, inhibits protein biosynthesis by transferring an ADP-ribosyl moiety to elongation factor 2 of eukaryotic cells. Exoenzyme S, encoded by the *exo* S gene, is also an ADP-ribosyltransferase that is secreted by a type-III secretion system directly into the cytosol of epithelial cells (Rumbaugh *et al.,* 1999). Las B elastase, a zinc metalloprotease encoded by the *la*s B gene attacks eukaryotic proteins such as

collagen and elastin, and destroys the structural proteins of the cell (Toder *et al.*,1994). While phospholipids are hydrolyzed by two phospholipases C encoded by *Plc H* and *plc N* genes (PLC-H and PLC-N, respectively). Alginate, encoded by *algD* gene, protects the bacterium from the host's immune response and from antibiotics (Wolska and Szweda, 2009).

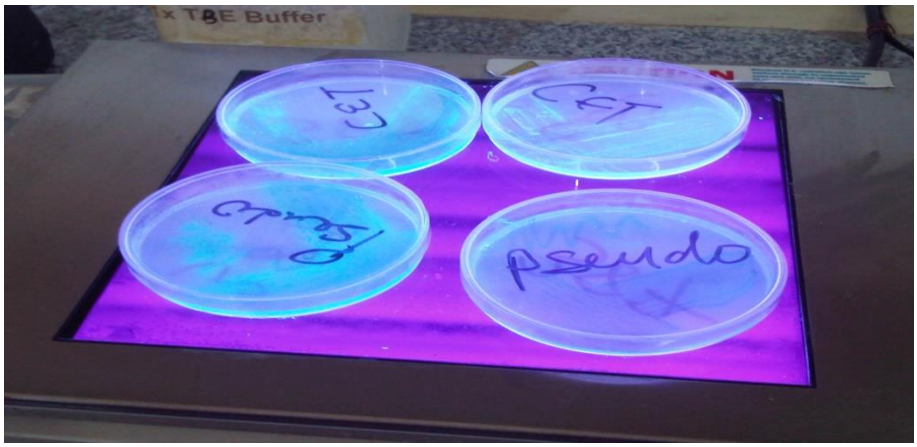
II. MATERIALS AND METHODS

Sample Collection

This study was carried out in Makurdi, the capital city of Benue State-Nigeria between the months of September, 2014 and April, 2015. A purposive selection consisting of pregnant women attending the ante-natal clinic was taken. Thus a total of 438 pregnant women attending the ante-natal clinic of the tertiary health institution Makurdi participated in this study. Information regarding each patient's medical and obstetric history was recorded on a predesigned proforma before collection of urine specimen was made. Information required on the proforma includes the age, parity, gestational age and symptoms relating to urinary tract infection. 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.

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^{o}C for 24hrs and then examined. Only plates with significant growth (i.e. at least 10^{5}cfu/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 illumination 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^{o}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 illumination for fluorescence. Suspected organisms were inoculated on nutrient agar and incubated for 24hrs at 37^{o}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.)



Petri dishes cultured on Cetrimide and Pseudocel agar showing fluorescent *Pseudomonas aeruginosa* viewed under UV light.

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 with 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 CLD with 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 8000 rpm 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 3 mins. This step was repeated by adding 500µl Buffer CWD3 for a third wash, centrifuged at 1400rpm for 3 mins. The column were 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).

Detection of Virulence genes by PCR

Six sets of primers (Forward and Reverse oligosequences) which target the following gene classes: alginate (*algD*), Las B elastase (*lasB*), entotoxin A (*toxA*), hemolytic phospholipase C (*plc H*), non-hemolytic phospholipase C (*plc N*) and exo-enzyme S (*exo S*) were used to amplify the respective genes from genomic DNA. Eppendorf Nexus Gradient Thermal Cycler was used for the DNA amplification following the modified protocol by Wolska & Szweda (2009) as follows: Bacterial cells were lysed to obtain genomic DNA for PCR as follows: three to five bacterial colonies were emulsified in 25 µl of a 0.25% sodium dodecyl sulfate-0.05 NaOH solution and boiled for 15 min. Then 600 µl of RNAse free H_2O was added to the mixture, and 5 µl of the diluted mixture was used in the PCR. Water was used as no template control (NTC), existing *E. coli* DNA was used as Negative Control (NC) and previously typed *P. aeruginosa* was used as Positive Control (PC).

Table 1: Primers used for PCR amplifications of virulence genes

Primer Name	Sequence (5'-3')	Product Size (bp)
Alg DF	CGTCTGCCGCGAGATCGGCT	313
Alg DR	GACCTCGACGGTCTTGCGGA	
Las BF	GGAATGAACGAAGCGTTCTCCGAC	284
Las BR	TTGGCGTCGACGAACACCTCG	
Tox AF	CTGCGCGGGTCTATGTGCC	270
Tox AR	GATGCTGGACGGGTCGAG	
Plc HF	GCACGTGGTCATCCTGATGC	608
Plc HR	TCCGTAGGCGTCGACGTAC	
Plc NF	TCCGTTATCGCAACCAGCCCTACG	481
Plc NR	TCGCTGTCGAGCAGGTCGAAC	
Exo SF	CGTCGTGTTCAAGCAGATGGTGCTG	444
Exo SR	CCGAACCGCTTCACCAGGC	

III. RESULTS

A total of 438 pregnant women urine samples were 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 1. The Analytical profile index (API) test kit was used to confirm identification of 28 *P. aeruginosa* isolates.

The amplification of the total DNA extracts using singleplex polymerase chain reaction to assess the prevalence of six virulence genes are presented in the figures below. Polymerase chain reaction (PCR) detected *alg* D in 2 (7.14%), *las* B in 3 (10.7), *tox* A in 9 (32.2%), *Plc* H in 1 (3.6%) and *Plc* N in 5(17. 9%) of the *Pseudomonas aeruginosa* DNA samples. (Figures 1, 2, 3 and 4). Singleplex PCR did not detect the presence of *exo* S gene from the 28 *Pseudomonas aeruginosa* DNA samples at all. The prevalent gene detected from the *P. aeruginosa* strains of the urine samples is the *tox*A gene, at 32.2%. Phospholipase C of the non- hemolytic type encoded by *plc N* gene occurred five times more than the hemolytic type encoded by *plc H* which was detected only in one sample. Apart from the *exo S* gene all studied genes were detected in one or more *Pseudomonal* strain.

Table 2: Distribution of uropathogens from pregnant women in Makurdi.

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)

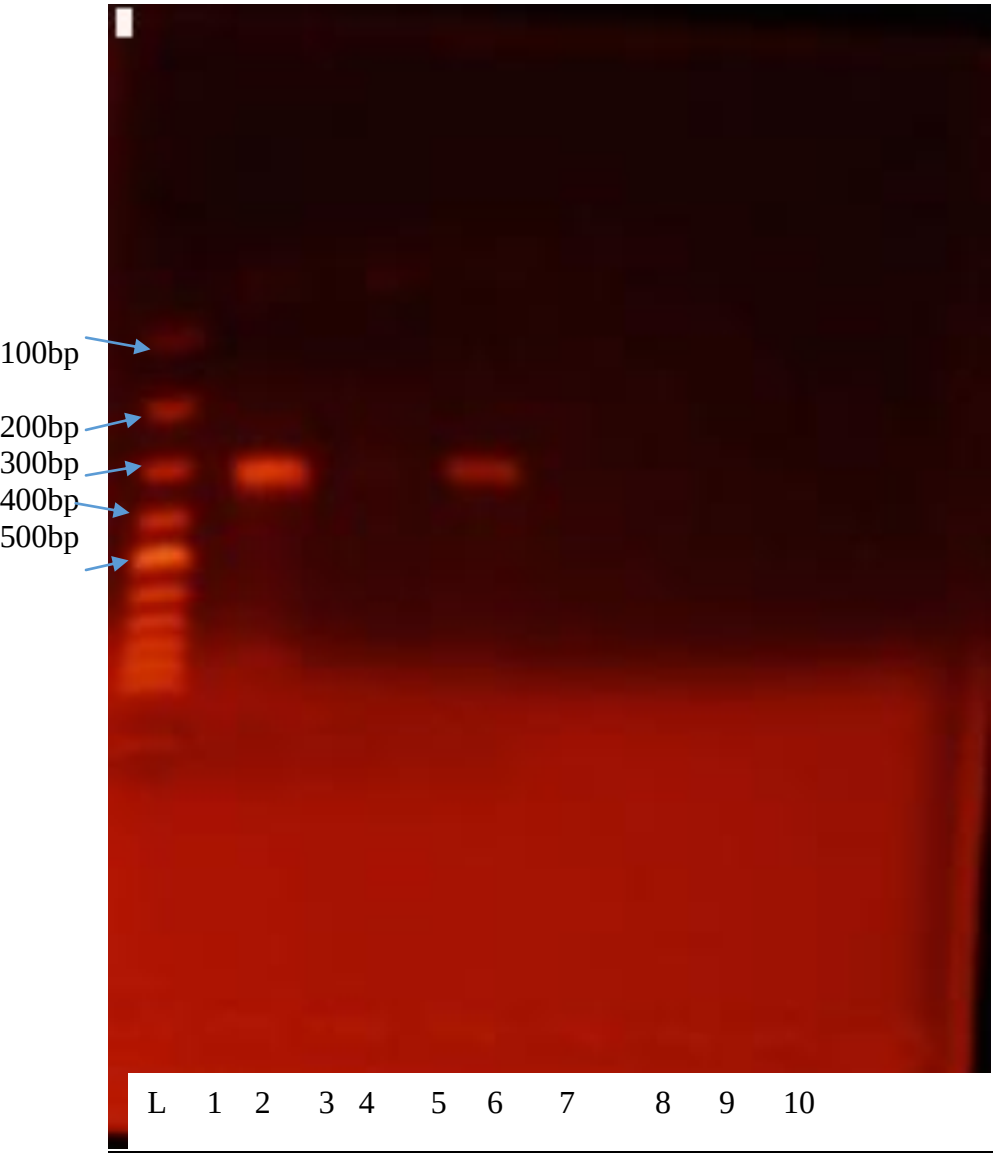


FIGURE 1: Agarose Gel Electrophoresis of PCR products amplified with a singleplex PCR method for the a*lg* D (313bp). Lane L: 100bp DNA Ladder, lane 2 and 5 ae positive for *alg* D virulent genes.

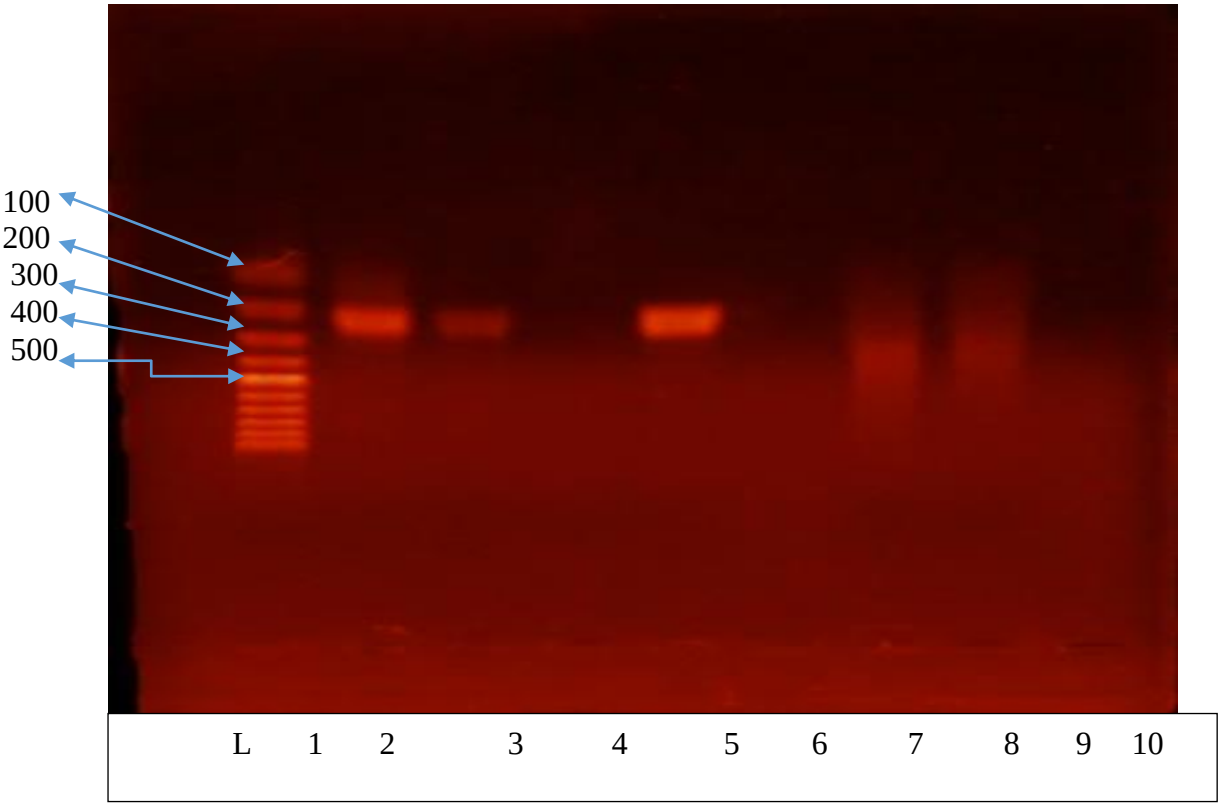


Figure 2: Gel electrophoresis of PCR products showing the presence of *las B* (284bp) virulent gene. Lane L: 100bp DNA ladder, Lanes 1, 2 and 4 are positive to the *las B* gene.

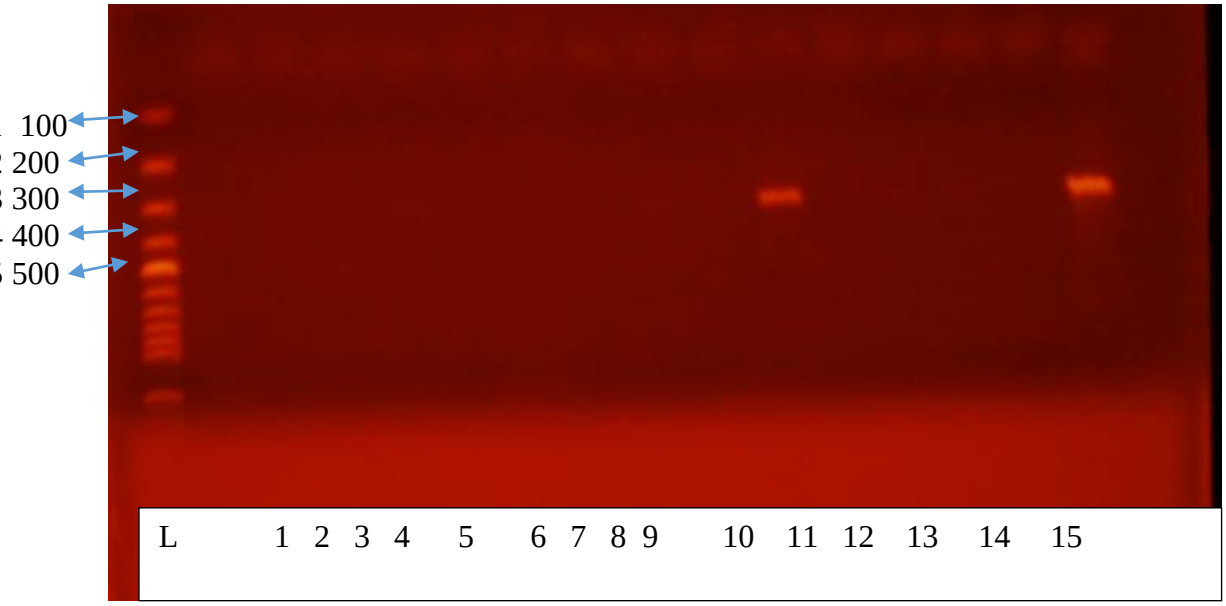


Figure 3: Agarose electrophoresis of PCR products amplified with a singleplex PCR method for the *tox*A(270bp) virulent gene. Lane L: 100bp DNA ladder, Lanes 10 and 15 are positive *tox*A virulent gene in*Pseudomonas aeruginosa*.

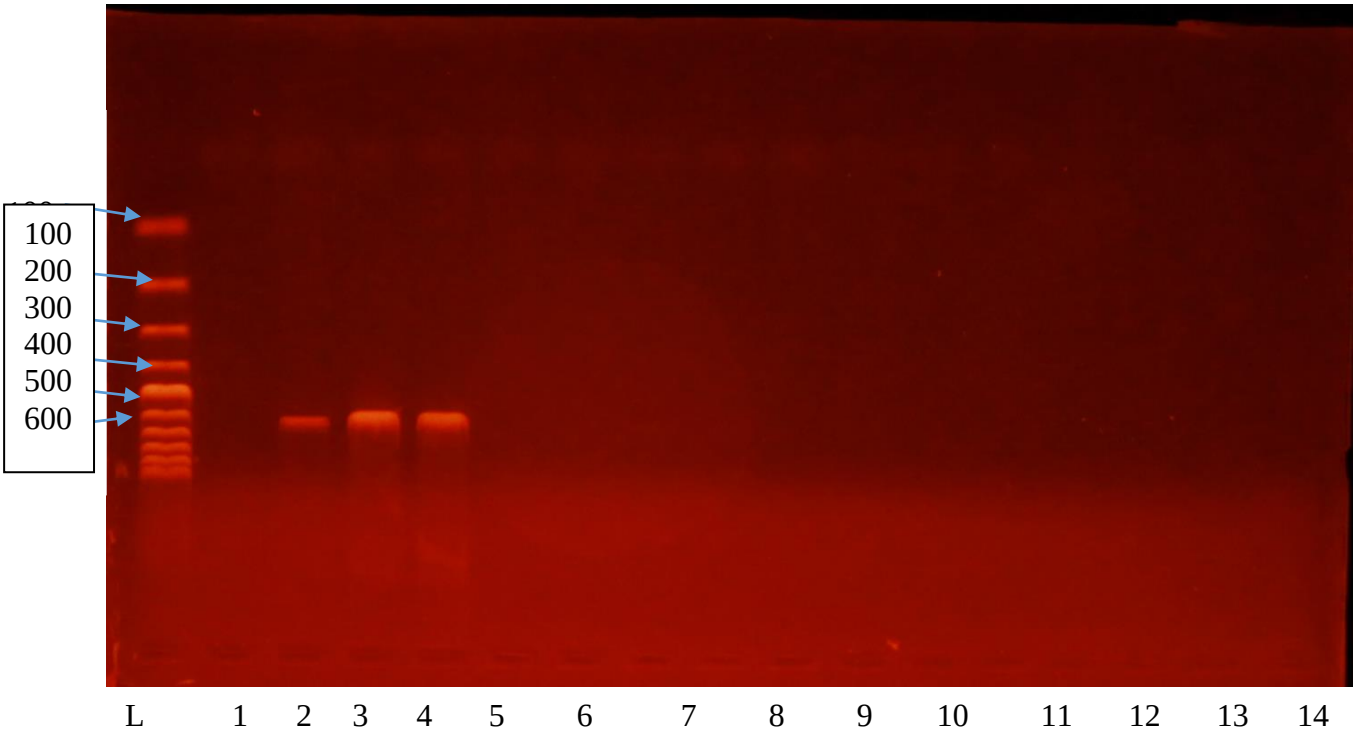


Figure 4: Agarose gel picture of virulence gene plc H at 608bp, L is 100bp DNA Ladder and lanes 2, 3, 4 are positive for plc H gene.

DISCUSSION

Pseudomonas aeruginosa has traditionally relied on phenotypic methods. This still is the most accurate standard when dealing with typical isolates of *P. aeruginosa*. (Khattab *et al*., 2015) *P. aeruginosa* isolates display unusual phenotypic reactions. Moreover, biochemical testing takes long time to perform and requires extensive hands- on work by the technologist, both for setup and for ongoing evaluation. Molecular methods have been reported to be superior to the phenotypic methods for identification of *P. aeruginosa* (Qin *et al*., 2003). Most studies report detection of virulent genes in *P. aeruginosa* from faeces, wound, bronchial washings, throat, ear, blood, sputum, cystic fibrosis patients among others. Not so much is reported from urine samples, although Wolska *et al*. (2009) carried out similar studies on 11 urine samples. In their study, all six virulence genes were detected in the urine cultures at about 90% in almost all genes except exo S which was detected in 54.5%. This findings are contrary to ours as detection was observed at lower percentages (3.6 - 32.2%) and even a zero percent detection of *exo S* gene. The variation and disparity in these findings may be as a result of differences in age, sex, sexual activity, pregnancy, the presence of genitourinary abnormalities, indwelling urinary catheters, and co-morbidities including diabetes mellitus and immunosuppressive conditions from two geographically different regions. In another carried out in Egypt by Khattab *et al.* (2003), they suggested that pathogenicity of *P. aeruginosa* is clearly multifactorial. *Las* B was one of the most important proteases of *P. aeruginosa* in their study all isolates examined harbored the *las* B gene from environmental and clinical isolates. The Zero percent detection of *exo* S gene agrees with findings of Lin *et al*. (2006) who rarely detected *exo* S gene in *P. aeruginosa* strains isolated from urine. According to them exo U gene of *P. aeruginosa* is the major contributor to cytotoxicity against mammalian cells. In this study exotoxin A encoded by the *tox* A gene was the prevalent virulence factor in *P. aeruginosa* urinary isolates, this agrees with studies of Rumbaugh *et al.* (1999) who reported that *P. aeruginosa* isolates recovered from patients suffering from urinary tract infections produced significant levels of exotoxin A. Mutation of *las* B gene reduces markedly *P. aeruginosa* invasion. Presence of the *las* B gene in some strains implies the importance of *las* B factor to survival of *P. aeruginosa*. The presence of both phospholipase C of the hemolytic and especially the non-hemolytic type encoded by *plc* H and *plc* N respectively are similar to those of Joly *et a*l. (2015) who suggested that in some bacterial infections, complex systems of coordinate regulation control the expression of virulence genes.

Conclusion:

This study showed the PCR detectection of virulence genes in the urinary tract of pregnant women. The presence of virulent genes could be a major cause of certain unknown complications associated with labor and child birth and this findings may also serve as epidemiological data necessary for health policies such as screening pregnant women for bacteriuria consequently predisposing them to pyelonephritis which may in turn lead to adverse outcomes for the baby and the mother, such as premature delivery, low birth weight infants, preeclampsia, hypertension, renal failure and fetal death.

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Conflict of Interest: The authors has no conflict of interest to declare.

REFERENCES

[1] 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 (2013) 1–10.

[2] Joly B., M. Pierre, S. Auvin, F. Colin, F.B. Guery and M.O. Husson. (2005). Relative expression of *Pseudomonas aeruginosa* virulence genes analyzed by a real time RT- PCR. Method during long infection in rats. FEMS Microbiol Lett. 243: 271-278.

[3] Khattab MA, Nour MS, ElSheshtawy NM (2015). Genetic Identification of Pseudomonas aeruginosaVirulence Genes among Different Isolates. J MicrobBiochemTechnol 7: 274-277. doi:10.4172/1948-5948.1000224.

[4] Lin H., S. Huang, H. Teng. D. Ji, Y. Chen and Y. Chen. (2006). Presence of the exo U gene Of *Pseudomonas aeruginosa* is correlated with cytotoxicity in MDCK cells but not with colonization in BALB /c mice. J. Clin. Microbiol. 44: 4596-4597.

[5] Narins, B. (2003). World of Microbiology and Immunology. Volume 1 and 2. Gale Group Inc., Michigan, 465-466.

[6] Rumbaugh K.P., J.A. Griswold and A.N. Hamood. (1999). *Pseudomonas aeruginosa* strains obtained from patients with tracheal, urinary tract and wound infections: variations in virulence factors and virulence genes. J Hosp. Infect. 43: 211-218.

[7] Schnarr, J. and Smaill, F. (2008). Asymptomatic bacteriuria and symptomatic urinary tract infections in pregnancy. European Journal of Clinical Investigation, 38 (S2): 50–57

[8] Tadesse, E., Teshome, M., Merid, Y., Kibret, B. and Shimelis, T. (2014). Asymptomatic urinarytract infection among pregnant women attending the antenatal clinic of Hawassa Referral Hospital, Southern Ethiopia. BioMed Central Limited Research Notes, 7:155.

[9] Toder, D.S. S.J. Ferrel, J.L. Nezezon, L. Rust and B.H. Iglewski (1994). Las A and Las B Genesof *Pseudomonas aeruginosa*: Analysis transcription and gene product activity. Infect. Immun. 62: 1320-1327.

[10] Van Delden C. abd B.H. Iglewski (1998). Cell- to- cell signaling and Pseudomonas aeruginosa infections. Emerg. Infec. Dis. 4: 551-60.

[11] Widmer, M., Gülmezoglu, A.M., Mignini, L. and Roganti, A. (2011). Duration of treatment for asymptomatic bacteriuria during pregnancy (Review). The Cochrane Collaboration, 12: 1-34.

[12] Wolska, K. and Szweda, P. (2009). Genetic features of clinical *Pseudomonas aeruginosa* strains. Polish Journal of Microbiology, 58(3): 255–260.

[13] Qin X, Emerson J, Stapp J, Stapp L, Abe P, et al. (2003) Use of real-time PCR with multiple targets to identify *Pseudomonas aeruginosa* and other non-fermenting gram-negative bacilli from patients with cystic fibrosis. J ClinMicrobiol 41: 4312-4317.