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Molecular Characterization of Waa Genes (waaA, waaC, wamB, wabG) Participate in Core Biosynthesis in P.mirabilis LPS

S.H. Ruaa and Mayyada F. Darweesh*

Abstract--- *LPS is a potential Proteus virulence factor that plays a key role in pathogenesis as well as in stimulating innate immune response. The structure of lipopolysaccharide differs from one bacterium to another, dependent on enzymes and gene products that can modify the basic structure of lipopolysaccharide in some bacteria, especially pathogens. So this study aimed to detection the present waa genes (waaA, waaC, wamB, wabG) that involve in biosynthesis of core LPS. 50 isolates of P. mirabilis were isolated from200 urine specimens taken from recurrent –urinary tract infections (UTI) patients attended to AL-Sadar Hospital. Specimens were cultured on specific media, and then bacterial isolates were identified depending on morphological, biochemical and VITK-2. The results showed that the. P. mirabilis comprise 11 (22%), 30(30%) and 9 (18 %) from recurrent UTI, kidney stone and Catheters samples respectively. The isolate that appeared multidrug resistance to all antibiotics used in this study were chosen to extracted lipopolysaccharide. Then this specimen was used for studies the genes responsible for synthesis of LPS,this specimen give positive result for waaA,waaC, wamB and wabG*

Keywords--- *P.mirabilis.LPS, Waagene, Molecular Characterization.*

I. INTRODUCTION

Proteus mirabilis, a swarming motile gram –ve bacteria one member of *Enterobacteriaceae* family, *P. mirabilis* is a common cause of urinary tract infections (UTI) in individuals with functional or structural abnormalities or with long-term catheterization, forms bladder and kidney stones as a consequence of urease-mediated urea hydrolysis so consider a serious complications arising from *P. mirabilis* infections (1).This bacterium is found more frequently than *Escherichia coli* in kidney infections so regard as uropathogenic bacteria(2). Known virulence factors, besides urease, are hemolysin, fimbriae, metalloproteases, and flagella (3). LPS is a potential *Proteus* virulence factor that plays a key role in pathogenesis. (4)

LPS is a central component of the outer membrane in Gram negative bacteria this molecule has a tripartite structure comprising the lipid A an endotoxic glycolipid that anchors LPS to the outer leaflet of the outer membrane which represent the proximal part of the LPS embedded in inner membrane with highly conserved structure, the core oligosaccharide which together with lipid A, contributes to maintain the integrity of the outer membrane, that connecting lipid A and O-antigen. The core domain is usually divided into inner and outer core on the basis of sugar composition and the core is the medial part with limited variation in the structure, finally, the O antigen polysaccharide that represents the distal part free in the external environment as a major surface antigen with hyper variable structure (5, 4).

S.H. Ruaa, Biology Dep. Faculty of Science, Kufa University, Najaf, Iraq.
Mayyada F. Darweesh, Biology Dep. Faculty of Science, Kufa University, Najaf, Iraq. E-mail: mayadajalala@yahoo.com*

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Most enzymes and genes coding for proteins responsible for the biosynthesis and export of lipopolysaccharide are shared by most Gram-negative bacteria based on genetic information. The structure of lipopolysaccharide differs from one bacterium to another, dependent on enzymes and gene products that can modify the basic structure of lipopolysaccharide in some bacteria, especially pathogens. These modifications are not required for survival, but are tightly regulated in the cell and closely related to the virulence of bacteria.(6). lipopolysaccharide synthesis required to blocks of genes involved in biochemical pathway (7).The LPS biosynthetic enzymes may be organized into clusters on the inner surface of the cytoplasmic membrane which are organized around a few key membrane proteins. The core region usually contains 10–15 monosaccharides. The O-antigen usually contains only a few monosaccharides, but can be repeated many times in LPS. Noncarbohydrate components are also found in these regions.(8)

the core regions of *P. mirabilis* of LPS consist of several genes as in other *Enterobacteriaceae* genes were clustered in the so called *waa* region (9).Four of the genes included *waaA, waaC, wabG* involved in synthesis of LPS and identification of two new heptosyltransferases (*WamA* and *WamC*),a galactosyltransferase (WamB), and an *N*-acetylglucosaminyltransferase (WamD). most of these genes were found in *waa* gene cluster (7)

II. MATERIALS AND METHODS

1-Bacterial Characterization

A total of 200 urine specimens were collected under aseptic condition from patients attending to Al-Sadar Medical City in AL-Najaf province were inoculated on MacConkey agar and Blood agar and incubated at 37 C° for 24 h. The morphological characteristics of the colonies including size, shape, colour, were recorded, the suspected *Proteus* were relevant by biochemical test (10), finally confirmed by using Vitek-2 Compact (Bio Mérieux,France,).

2- Extraction of lipoopolysacharide (LPS)

A- Cell Preparation

Proteus mirabilis isolate were cultured all night in flask containing 25 ml of LB broth (for bacterial activation) at 37ºC for 18 hours. The fresh cultures then inoculated 3.5 L of LB broth suspended in 500ml conical flasks containing 200 ml broth. Incubated at 37ºC for 24 hours with shaking at 150 rpm. Following centrifugation at 3000 rpm for 15 minutes, the pellet was washed twice with phosphate buffer. Cells were suspended in phosphate buffer containing 0.5% formalin (pH= 7.2) and were kept at 4ºC for 18 hours. The precipitate were centrifuged at 3000 rpm for 15 minutes and washed with phosphate buffer. Finally, cells were dried using cold acetone by ten times the sample's volume (11).

B- Lipopolysaccharide Extraction

The *Proteus mirabilis* LPS was extracted from MDR isolate by the hot EDTA method given by (12).

3- Molecular Studies of Proteus mirabilis

A- Extraction of Bacterial DNA

Genomic DNA extraction was done according to protocol of (13) used Tm Mini DNA Bacteria Kit (Geneaid) The extracted genomic DNA is checked by using Nano drop spectrophotometer which measure DNA concentration (ng/µl) and checks the DNA purity by reading the absorbance at (260/280) nm.The method described by (14)

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B- PCR Assay

All isolates of *Proteus mirabilis* were screened for four of the genes (*waaA*, *waaC*, *wamB* and *wabG*) to detection pathway for lipopolysaccharides synthesis as shown in (Table 1-1). These primers synthesized by Bioneer (Korea)

C- Preparing the Primers Suspension

The oligonucleotide primers were resuspended depending on manufacturer's instructions Bioneer (Korea)

Table 1-1: The forward and reverse of primers

No	primer	Sequencing	Product size
1	*waaA*	F : CAGGCGCAAAAGTCCGTATC R : TTCCCGCCCATAAAACTTCG	618
2	*waaC*	F : TGTTCAGCATCGCCTTTACG R : AATCCGCTTTAGTGCCGTTC	691
3	*wamB*	F : ACCCCGTTTTCAGCAACTTT R : TAACTGAAGGTGAGCGTCGT	611
4	*wabG*	F : GTTTTGTTTCTCGCGCACTG R : ATCGCCTCAATAGCAGCTCT	619

D- PCR Amplification

The method which was described by (15) PCR products were resolved on 1.5% agarose gels stained with 5 µl ethidium bromide, and photographed with UV illumination.(table1-2)

Table 1-2: PCR program that apply the primer.

Steps	Temperature	Time	No. of cycles
Initial Denaturation	95 ^{o}C	5 min	1
Denaturation	95 ^{o}C	30 sec	30
Annealing	57 ^{o}C	30 sec	
Extension	72 ^{o}C	45 sec	
Final extension	72 ^{o}C	10 min	1

III. STATISTICAL ANALYSIS

The results are presented as means ± standard error (S.E) and statistical analyzed using one- way analysis of variance (ANOVA) test. Using Graph pad prism 5.04. $P < 0.05$ was considered significant.

IV. RESULTS

1-Characterization of P.mirabilis

A total of 200 urine sample from patients suffering recurrent UTI have been collected and tested to isolate *P.mirabilis*.Only 50 (25%) sample gave growth of *P.mirabilis* depending on characteristics of the microscopic, morphological, biochemical tests and Vitek 2system as in (Fig-1).Colonies of *P. mirabilis* appeared pale, yellow, and non-lactose fermenters on MacConkey agar. While on blood agar *P. mirabilis* isolates showed the swarming phenomenon as well as the fish odor and cause β-hemolysis colonies on blood agar. (16). All members of *Proteus spp* were negative for oxidase test, VP and indole test, while give positive result for MR test (17).

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2-A-Extraction of Lipopolysaccharide

The results of extracted LPS appeared that the present employed method yielded a bacterial mass of 20 gm dry weight bacteria and obtained 130 mg of crude lipopolysaccharide.

B- LPS extraction and chemical analysis

The results of chemical analysis of LPS in 1 ml of crud and partial purified LPS showed that the carbohydrate concentration were 159 µg/ml and 230 µg/ml respectively, while protein concentration recorded 40 and 23 µg/ml respectively.

3- Genotyping of lipopolysaccharide

The results of *WaaA, WaaC, wamB* and *wabG* gene are detection by PCR technique that illustrated the isolate were possessed biosynthesis genes for LPS according to product size of the primer(fig -1)

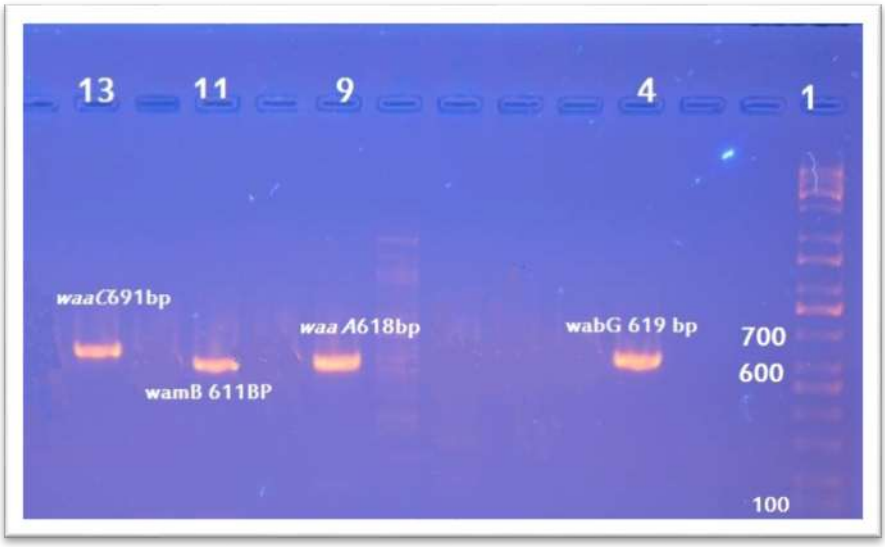

Figure 1: Gel electrophoresis and PCR product of LPS genes, Ladder (100-1000bp). waaC gene 691 bp, wamB gene 611 bp, waaA gene 618 bp and wabG gene 619 bp.

These results were similar to (18) who able to identify the functions of the genes found in the *waa* gene cluster from *P. mirabilis* strains that required for the biosynthesis of the LPSs.

So (19) identify the *waa* clusters gene found in gram negative bacteria. LPS is one of the major structural and immunodominant molecules of the outer membrane. It consists of three domains: lipid A, core oligosaccharide, and O-specific antigen. The genes that involved in LPS core biosynthesis in most *Enterobacteriaceae* are usually found as clustered in a region of the chromosome, the *waa* (*rfa*) gene cluster as in *K.pneumia* (18). In other species these genes may be not clustered may be distributed between several region as in *yersinia pestis* or *proteus mirabilis* although one common core biosynthetic gene (*wabO*) was found outside this cluster. (9)

The core-OS of these strains share a common heptasaccharide fragment that includes a3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo) disaccharide, a L-*glycero*-α-D*manno*- heptosepyranose (LD-Hep*p*) trisaccharide, and one residue each of Dglucose (Glc), D-galacturonic acid (GalA) and (20)found the *waa* gene cluster functions involved

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in the biosynthesis of its inner and outer core LPS, in the inner core the *waa* gene cluster contains two conserved regions; One is in the proximal end of the cluster with three conserved genes, gmhD, *waaF* and *waaC*, encoding the last enzyme in the biosynthesis of l,d-Hepp, lglycero-d-manno-heptosyltransferaseII and l-glycerod- manno-heptosyltransferase I, respectively (21). *waaA* gene a Kdo transferase, utilizing the activated sugar nucleotide CMP-Kdo by the sequential transfer to lipid A of one to two residues of Kdo by the CMP-Kdo:lipid A Kdo bifunctional transferase (*WaaA*) (22). In *E. coli* the *waaA* operon is transcribed divergently from the 10- gene operon, beginning with *wabQ*, which encodes enzymes involved in synthesis and modification of the LPS core oligosaccharide and three residues of L,D-heptose by ADP-heptose-heptosyltransferases I, II, and III ; *WaaC, WaaF,* and *WaaQ* (23). It is depending on the bacteria species could be mono,bi, tri or tetra-functional transferring either one, two, three, or four Kdo molecules, respectively, to the tetra-acylated Lipid A precursor, Lipid IVA.(24, 25). The *WaaA* would remain bound to the lipid acceptor until the correct number of Kdo glycosylation events occur where the glycosylated lipid is then released.(19). (26) clarified that the heptosyltransferase *WaaC* and *WaaF* genes are implicated in the transfer of L-D-HepI and L-D-HepII to the principal chain of the LPS core; it has also been confirmed that the configuration of L,D-Hep residues in the core is established by the specificity of these heptosyltransferases.

(18) Found the *WaaA* homologues *of P. mirabilis* showed high levels of amino acid identity and similarity to *E. coli WaaA* (75% and 85%). The *WaaC, WaaF,* and *WaaQ* homologues showed high levels of identity and similarity to *K. pneumoniae* homologues *WaaC* (68% and 80%), *WaaF* (75% and 83%), and *WaaQ* (54% and 69%)

The outer core of LPS is synthesized through the sequential transference of the different residues from nucleotides-activated precursors. The process is due to the action of several glycosyl- transferase that act on the cytoplasmatic surface of the internal membrane, where is available not only the acceptor but also the activated precursors (27).

The other gene of *waa* cluster revealed in the synthesis of outer core,the *WaaG* protein has been identified as the glucosyltransferase involved in the transfer of the first outer core LPS residue in *E. coli* and *S. enteric* serovar typhimurium. *K. pneumoniae orf8*-encoded protein showed significant similarity to the *WaaG* protein from *E. coli* ; so this *K. pneumonia* and *P.mirabilis* gene was named *wabG* (28)

(18) mention that the wabG shared between *Proteus* and *Klebsiella* showed that the *wabG*, *wabH*, and *wabN* homologues are transcribed in the same additional genes named *wamB* and *wamC* (*wam* stands for *wa* genes from *P. mirabilis*) are inserted between *wabH* and *wabN* homologues *wamA*, *wamB,* and *wamC*) were expected to be involved in outer-core completion. A BLAST search of the putative proteins encoded by *wamA* and *wamC* showed high similarity and identity to heptosyltransferases function from *P. mirabilis*and they are candidates for the transfer of the two outer-core D,D-Hep residues.

In conclusion the biosynthesis of LPS a complex process represent by chemical pathway required several genes that may present as cluster genes in same chromosome or may distribution in several region.Structure of *Proteus mirabilis* LPS similar to genes that involve in *K. pneumonia* LPS as well as *E. coli* LPS.

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REFERENCES

[1] Alelign, T., and Petros, B. (2018). Kidney Stone Disease: An Update on Current Concepts. *Hindawi J. Advances in urology*, Volume 2018, P12

[2] Yun, K. W., Lee, M. K., Kim, W., and Lim, I. S. (2017). uropathogenic Escherichia coli ST131 in urinary tract infections in children. *Korean J. of pediatrics*, 60(7), 221-226.

[3] Schaffer, J. N., and Pearson, M. M. (2015). Proteus mirabilis and urinary tract infections. *J. Microbiology spectrum*, 3(5).

[4] Whitfield,C., and Trent, M. S. (2014).Biosynthesis and export of bacterial lipopolysaccharides *J. Annual review of biochemistry*, 83, 99-128.

[5] Clifton, L. A., Ciesielski, F., Skoda, M. W., Paracini, N., Holt, S. A., and Lakey, J. H. (2016). The effect of lipopolysaccharide core oligosaccharide size on the electrostatic binding of antimicrobial proteins to models of the gram negative bacterial outer membrane. *J. Langmuir*, *32*(14), 3485-3494.

[6] Wang, X., and Quinn, P. J. (2010). Lipopolysaccharide: Biosynthetic pathway and structure modification. *J. Progress in lipid research*, *49*(2), 97-107.

[7] Aquilini, E. (2013). Lipopolysaccharide (LPS) core biosynthesis in" *Proteus mirabilis*". *Thesis, Facultad de Farmacia, Universidad de Barcelona*, p24-80

[8] James P. Coleman, C. Jeffrey Smith, (2007), Microbial Cell Wall Synthesis and Permeability, *x Pharm: The Comprehensive Pharmacology Reference* page 1-4

[9] Kaszowska, M., de Mendoza-Barberá, E., Maciejewska, A., Merino, S., Lugowski, C., and Tomás, J. M. (2017). The Complete Structure of the Core Oligosaccharide from Edwardsiella tarda EIB 202 Lipopolysaccharide. *International j of molecular*

[10] Dharmadhikari, S.M., and A.S. Peshwe. (2009). Molecular level studies on multiple antibiotic and serum resistance in UTI pathogens. *Indian J. Biotechnol*. 8:40-50 7

[11] Silipo, A., Lanzetta, R., Amoresano, A., Parrilli, M. and Moliaro, A.(2002a). Ammonium hydroxide hydrolysis: a valuable support in the MALDI-TOF mass spectrometry analysis of lipid A fatty acid distribution. *J. Lipid Res*.. 43: 2188-2195.

[12] Chandan, V. and Fraser, A.D. (1994). Simple extraction of Campylobacter lipopolysaccharide and protein antigens and production of their antibodies in egg yolk. *Inter. J. Food Micriobiol*. 22:189-200.

[13] Li, B., Carey, M., and Workman, J. L. (2007). The role of chromatin during transcription. *J. Cell*, *128*(4), 707-719.

[14] Sambrook J, and, Russell DW. (2001) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, New York;. pp: 370-380.

[15] Joshi, R.M. and Albert, M.J. (2009). Hybrid ELtor *Vibrio cholerae* O1. *J.Emerg. Infect*. 11: 1879-1880

[16] Holt, J.G., Krieg, N.R., Sneath, H.A., Staley, J.T. and Williams, S.T.(1994). Bergey's Manual of Determinative Bacteriology. 9th Ed. Williams and Wilkins.USA.

[17] Bharti, A. and. Arora, D.R. (2008). Practical Microbiology, Maharagja Agrasen Medical Collage, Agroha, Hisar, Haryana (India). ISBN: 978-81-239-1595-1.

[18] Aquilini, E., Azevedo, J., Jimenez, N.,Bouamama, L., Tomás, J.M., and, Regué, M.(2010) Functional Identification of the Proteus mirabilis core lipopolysaccharide biosynthetic genes. *J. Bacteriol*. 192, 4413–4424.

[19] Pratt, A. (2017). Functional Analysis of Early Core Region Modification in the LPS of Gram-negative Bacteria. *Thesis, University of Michigan* p23-80

[20] Vinogradov, E., Z. Sidorczyk, and Y. A. Knirel. (2002). Structure of the lipopolysaccharide core region of the bacteria of the genus *Proteus. Aust. J. Chem*. 55:61-67.

[21] Grizot, S., Salem, M., Vongsouthi, V., Durand, L., Moreau, F., Dohi, H., and Ducruix, A. (2006). Structure of the *Escherichia coli* heptosyltransferase *WaaC*: binary complexes with ADP and ADP-2-deoxy-2-fluoro heptose. *J. of molecular biology*, *363*(2), 383-394.

[22] Schmidt, H., Hansen, G., Singh, S., Hanuszkiewicz, A., Lindner, B., Fukase, K., Woodard, R. W., Holst, O., Hilgenfeld, R., Mamat, U., and Mesters, J. R. (2012) Structural and mechanistic analysis of the membrane-embedded glycosyltransferase WaaA required for lipopolysaccharide synthesis, *Proceedings of the National Academy of Sciences of the United States of America* 109, 6253-6258

[23] Jiménez, N., Senchenkova, S. N., Knirel, Y. A., Pieretti, G., Corsaro, M. M., Aquilini, E., and Tomás, J. M. (2012). Effects of lipopolysaccharide biosynthesis mutations on K1 polysaccharide association with the *Escherichia coli* cell surface. *J.of bacteriology*, *194*(13), 3356-3367.

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[24] Gattis, S., Chung, H., Trent, M., and Raetz, C. (2013) The origin of 8-amino-3,8-dideoxy-D-manno-octulosonic acid (Kdo8N) in the lipopolysaccharide of *Shewanella oneidensis*, *The Jof biological chemistry 288*, 9216-9225.

[25] Zachman-Brockmeyer, T. R., Thoden, J. B., and Holden, H. M. (2016) Structures of KdnB and KdnA from *Shewanella oneidensis*: Key Enzymes in the Formation of 8-Amino-3,8-Dideoxy-D-Manno-Octulosonic Acid,*J. Biochemistry 55*, 4485-4494

[26] Aquilini, E.; Tomás, J.M.(2015) Lipopolysaccharides (Endotoxins). In Reference Module in Biomedical Sciences; Elsevier: Amsterdam, The Netherlands,; in press.

[27] King, J. D., Kocíncová, D., Westman, E. L., and Lam, J. S. (2009). Lipopolysaccharide biosynthesis in *Pseudomonas aeruginosa*. *J. Innate immunity*, *15*(5), 261-312.

[28] Regué, M., Climent, N., Abitiu, N., Coderch, N., Merino, S., Izquierdo, L., and Tomás, J. M. (2001). Genetic characterization of the *Klebsiella pneumoniae* waa gene cluster, involved in core lipopolysaccharide biosynthesis. *J. of bacteriology*, 183(12), 3564-3573.

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