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Research Article

LIPOPOLYSACCHARIDE PROFILING OF ESBL PRODUCING *PROTEUS* ISOLATES IN RAW MILK AND MILK PRODUCTS

***Syed Waseem Sajad**
Department of Microbiology, Singhania University, Jhunjhunu, Rajasthan India
**Author for Correspondence*

ABSTRACT
The lipopolysaccharides are major constituent of cell wall of gram-negative bacteria and are recognized as endotoxin of gram-negative bacteria. The bacterial lipopolysaccharide typically consists of hydrophobic domain known as lipid A, a non-repeating core oligosaccharide and a distal polysaccharide (O-antigen). ESBL producing *Proteus* isolates were used for isolation of lipopolysaccharides by using Tri-reagent method. In this method it was found that ester and phosphoester bonds of lipid A are easily hydrolyzed. The SDS-PAGE was used for analysis of LPS of different strains. The analysis by SDS-PAGE, followed by Silver staining was used to characterize LPS individually and this silver staining was found to sensitive up to ng concentration of LPS. The LPS of different strain produced dark "stair case" pattern of bands due to carbohydrate chain length variation of O-antigen portion. The silver staining method for LPS was also standardizing by using different concentration of LPS. The observed band pattern was found to be as such as band pattern reported by Tsai and Frasch, (1982). Each band patterns represent a molecule of LPS with increasing unit of O-polysaccharide. Mainly two types of band pattern were observed. Some intense and highly concentrated bands were observed at upper gel layer and some intense bands which were not clearly distinguished were observed at lower surface. The bands at bottom were supposed to have short chain O-polysaccharide and those at the top have long chain of O-polysaccharide. Appearance of dark bands of LPS on SDS-PAGE revealed that isolates were highly pathogenic.

Key Words: *Lipopolysaccharide, Endotoxin, Gram-negative Bacteria, Lipid-A, Electrophorosis*

INTRODUCTION
Lipopolysaccharide of most strains of gram-negative bacteria is heterogeneous. A lipopolysaccharide (LPS) is a large molecule that contains both lipid and a carbohydrate. It comprises three parts: polysaccharide (O) side chains; core polysaccharide: and lipid A. Lipid A contains unusual fatty acids (e.g. hydroxyl-mysteric acid) and is inserted into outer membrane while the rest of LPS projects from the surface. Core polysaccharide contains usual sugars (e.g. KDO and heptulose). It contains two glucosamine sugar derivatives each containing three fatty acids with phosphate or pyrophosphate attached. The core polysaccharide is attached to lipid A, which is also a part responsible for toxicity of gram-negative bacteria. However it is also an active immunomodulator, able to induce non-specific resistance to both bacterial and viral infections. The polysaccharide side chain is referred as O-antigen of bacteria. O side chain (O antigen) is also a polysaccharide chain that extends from the core polysaccharide. The composition of O side chain varies between different gram-negative bacterial strains. O side chains are easily recognized by antibodies of the host; however, the nature of side chain can easily be modified by gram negative bacteria to avoid detection. LPS are essential for survival of gram negative bacteria, providing a permeation barrier for harmful substances. They are highly immunogenic, and stimulate the production of endogenous pyrogen interleukin-1 (IL-1) and tumor necrosis factor (TNF). LPS also increase the negative charge of cell wall and help stabilizing the overall membrane structure. LPS acts as the protypical endotoxin, because it binds the CD14/TKR4/MD2 receptor complex, which promotes the secretion of pro-inflammatory cytokines in many cell types. LPS of bacteria are potent stimulators of immune system, including acute proinflamatory responses and septic shock. In *Proteus* LPS prevents from bactericidal effects of complement protein deposition. There are different mechanisms

for LPS mediated compliment activation (Vukajlovich *et al.*, 1992). The antibody-independent classical pathway is mediated by the lipid A portion. Activation of alternate pathway requires the polysaccharide moieties of LPS, core oligosaccharide and O-antigen polysaccharide. Based on O- antigen of LPS three species of *Proteus, P.vulgaris, P. penneri, and P.mirabilus* were classified into serogroups (Knirel *et al.*, 1992). A peculiar feature of *Proteus* O- specific polysaccharide is their acidic nature due to the presence of hexuronic acids, their amino acids, phosphate groups and other acidic non sugar components, which often serve as epitopes recognized by specific antibodies. Most *Proteus* O-antigens have a unique structure, but some of them possess marked structural similarities to another, which accounts for the serological cross reactivates of strains. Some *Proteus* O-antigens are structurally related to O-antigens or capsular polysaccharides of taxonomically distinct bacteria (Knirel *et al*., 1992).The monomer molecular weight of this lipopolysaccharide is approximately 10,000 Daltons.

MATERIALS AND METHODS

A. Isolation of Lipopolysaccharide by using TRI-REAGENT method

Tri- Reagent Method

Principle

The major components of commercial RNA isolation reagent, Tri reagent are phenol and Guanidium thiocynate in aqueous solution. The bacterial cell membranes are disrupted with Guanidium thiocynate which eliminate the need for mechanical cell disruption or heating. Negative ion quadruple trap and matrix-assisted laser disruption/ ionization time of flight, mass spectrometry fatty acid composition analysis by capillary gas chromatography, total and free phosphate by UV spectrophotometer and Sodium dodecyle sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that LPS and lipid A isolated using Tri - Reagent method approach were cleaner and suffered less degradation through loss of phosphate and fatty acyl side chain from lipid A.

Reagents for Tri –Reagent Method

1) Tri – Reagent:

1M Guanidium thiocynate = 0.23 in 2 ml
Phenol = 200 µl

Mixed above these two contents to make Tri – Reagent

2) Chloroform
3) 0.375 M $MgCl_2$: Prepared by dissolving 3.81 g of $MgCl_2$ in 50 ml of water
4) 95% ethanol : This solution should be stored at -20°C

Procedure

1. Lyophilized bacterial cell (1-10mg) were suspended in 200µl of Tri-Reagent
2. The cell suspensions were then incubated at room temp. for about 10 -15 min for complete cell homogenization.
3. After incubation, 20µl of Chloroform per mg of cells were added to create a phage separation.
4. The mixtures were then vigorously vortexed and incubated at room temp. For additional 10 min then centrifuged at 12000 rpm for 10 min to separate the aqueous and organic phage.
5. The aqueous phage was transferred into a new 1.5 ml centrifuge tube.
6. Distilled water (100µl) was added to organic phage.
7. The mixture was vortexed and incubated at room temp for 10 min and centrifuged at 12000 rpm for 10 min.
8. Nucleic acids were removed by reconstitution of the LPS-enriched extracts to 10 mg/ml in 0.1M Acetate buffer with 0.02% $MgSO_4$ and 0.4% Chloroform and digestion with RNase and DNase by incubation at 37^{o}C over night. Contaminating protein was then removed by the addition of Proteinase K, followed by heating at 60^{o}C for 1 hr. and incubation overnight at 37^{o}C.
9. Crude Tri- reagent extracted LPS was dissolved in 500µl of 0.375 M $MgCl_2$ in 95% ethanol stored at -20°C. Followed by centrifugation at 12000 rpm for 15 min.

10. The pellets were suspended in 200µl of distilled water and lyophilized to give fluffy white solid LPS totally 15-20% of starting material dry wt. Then run this sample in to SDS-PAGE electrophoresis.

B. Analysis of Lipopolysaccharide of Different Strains by SDS-PAGE Electrophoresis

Polyacrylamide Gel Electrophoresis

The rate of migration of a compound depends on its net charge, size, shape and applied current (Hames and Rick, 1990). The proteins are dissociated into their constituent's subunits using anionic detergent such as Sodium Dodecyle Sulphate (SDS) or sodium lauryl sulphate. The intrinsic charges of polypeptides are insignificant compared to negative charges provided by bound detergent. In case of reducing SDS-PAGE, a Thiol reagent such as β- Mercaptoethanol is included which cleaves the disulphide bonds. So in this method a protein sample is boiled in presence of excess SDS and β-Mercaptoethanol to denature proteins to their individual polypeptides. SDS polypeptide complex (1.4 SDS: 1 protein) have net negative charge and migrate towards anode at rates based solely on size or molecular wt. of peptides.

Reagents

1) Acrylamide- bisacrylamide stock solution:

Acrylamide = 30.0g
Bisacrylamide = 00.8g

Then dissolved Acrylamide and Bisacrylamide in water and make final volume to 100 ml. Filtered the solution through Whatman No.1 filter paper and stored in brown bottle at 0-4°C. This was stable for one month.

2) Stacking gel Buffer stock (Tris-HCL, pH-6.8)

Tris = 6.0 g
1M HCl = 48.0 ml

Then adjust pH to 6.8 and make, its final volume to 100 ml with water. Filtered through Whatman No.1 filter paper and stored at 0-4°C.

3) Resolving gel Buffer stock (Tris-HCL, pH-8.8)

Tris = 36.3.g
1M HCl = 48.0 ml

Then adjust pH to 8.8 and make, its final volume to 100 ml with water. Filtered through Whatman No.1 filter paper and stored at 0-4°C.

4) 1.5% Ammonium Per Sulphate (APS): Prepare by dissolving 0.15g of APS in 10 ml water. This reagent should be prepared fresh just before use.

5) N,N,N',N'-Tetra Methyl Ethylene Diamine (TEMED)

6) Reservoir buffer or electrode buffer (Tris- glycine, pH-8.3)

Tris = 3.0.g
Glycine = 14.4 g
SDS = 1.0 g

Then adjust pH to 8.3 and make, its final volume to 1L with water.

7) SDS (10% w/v): Dissolved 1g SDS in 10 ml of distilled water. Stored it at room temp.

8) Sample buffer 2X:

1M Tris-HCL, pH – 6.8 = 3.0.g
Glycerol = 20.0 ml
SDS = 4.0 g
β- Merceptoethanol = 10.0ml
1% Bromophenol blue = 4.0 ml
SDS = 1.0 g

9) 0.01% Bromophenol blue solution: Then dissolved 10 mg of Bromophenol blue in 100ml of water.

Stock solution	Resolving gel or separating gel (12.5% ml of the sol.)	Stacking gel (2.5% ml of the sol.)
Acrylamide- Bisacrylamide	12.5 ml	2.5 ml
Stacking gel buffer stock solution (pH-6.8)	-	5.0 ml
Resolving gel buffer stock solution (pH-8.8)	3.75 ml	-
10%SDS	0.30 ml	0.20 ml
1.5% APS	1.5 ml	1.0 ml
Distilled water	11.95 ml	11.30 ml
TEMED	0.015 ml	0.015 ml
Total Volume	**30.0ml**	**20.0ml**

Procedure

a) Sample preparation

First mixed the sample with equal volume of sample buffer then boiled the mixture for 3 min's. in the boiling water bath and cooled to room temp.

b) Preparation of Slab gel

1. Thoroughly cleaned and dried glass figures were assembled in gel casting assembly. Sealed the two glass figures with the help of tygon tubing, clamp them and placed the whole assembly in upright position.
2. Then mixed the various components of resolving gel as indicated in above table except for SDS, APS and TEMED. Degas the solution for 1 min. using a water pump and then added the above remaining components of gel.
3. Then mixed gently and poured the gel solution into the mould in between the clamped glass figures taking care to avoid entrapment of any air bubble. Overly distilled water on top as gently as possible and leave for 30 min. for setting of gel.
4. When the gel had polymerized, removed the water layer and rinsed the gel surface with stacking gel buffer.
5. Then mixed the stacking gel components and poured the stacking gel and immediately inserted the supplied plastic comb in stacking gel. Then allowed the gel to polymerize for about 20 min.
6. After the staking gel polymerization, we removed the comb without damaging the shape of wells and cleaned the wells by flushing with electrode buffer using a syringe.
7. Removed the tygon tubing and installed the gel figure assembly into the electrophoretic apparatus.

c) Electrophoresis of sample

1. Loaded 10-20 µl of sample (100-200 µg protein) in the sample wells. Also loaded molecular weight marker in one of the wells.
2. Switch 'ON' the current, maintain it at 10 -15 mA for initial 10- 15 min. until the samples have traveled through the stacking gel. Then increase the current to 30 mA until Bromophenol blue dye reached near the bottom of gel slab. This may require 3-4 hrs.
3. After the electrophoresis had completed, Turned 'OFF' and disconnected the power supply and carefully removed the gel slab from in between the glass figures.
4. Placed the gel in a trough containing staining solution for 3-4 hrs or keep it for staining overnight. Destained the gel with destaining solution till the clear background of gel is obtained.

C. Characterization of Lipopolysaccharide by Silver staining

Silver Staining

Principle

Ag^+ ions complex with bases of DNA and sulfhydryl and carboxyl group of proteins. Ag^+ complexed with DNA or Protein is selectively reduced to metallic, black and visible Ag. Ag+ in the solution is more

slowly reduced then is complexed Ag+. Coloration of Silver stained protein bands is caused by light scattering from Silver stains of characteristic sizes. DNA and proteins are not modified. When the concentration of proteins or DNA is very low (ng), we refer to Silver staining. It is most sensitive staining method known for protein and DNA Fragments (Tsai and Frasch, 1982).

Reagent

1. Firstly prepared the Silver Fix Solution: 50% Methanol + 12% Acetic acid + 0.02% Formaldehyde.
2. 100x Sodium Thio Sulphate : 2% (w/v) Sodiumthiosulphate (covered with aluminum foil)
3. 10x Sodium Carbonate : 0.566 M Sodium Carbonate (6%w/v)
4. 50 x Silver Nitrate: 0.588 M Silver Nitrate (10%w/v) (Covered with aluminum foil.)
5. Silver solution: 4 ml of 50 x Silver Nitrate +150 µl, 35% Formaldehyde in 200 ml of distilled water. (Covered with aluminum foil.)
6. Silver developing solution: 40 ml. 10X Sodium Carbonate +20µl, 55% Formaldehyde +8µl. 100x Sodium Thio Sulphate in 200 ml of double distilled water.

Procedure

1. Fixed the gel at least for 1 hr but preferably overnight in Silver fixing solution.
2. Washed the gel three times for 20 min in 50% alcohol.
3. Took the gel for exactly 1 min in 1x Sodium Thio Sulphte.
4. Washed the gel three times for 20 sec in distilled water.
5. Gently agitated the gel in Silver solution for about 30 min in dark.
6. Washed the gel twice for 20 sec in distilled water.
7. Washed vigorously with 1x Sodium Carbonate (for 5 min. with shaking)
8. Developed the gel in Silver developing solution (2-3 hrs or more until black bands appeared)
9. Washed in water (5-15 min.).
10. Stopped the reaction by adding 5% Acetic acid for 2 min.
11. Stored the gel in 1% Acetic acid.

RESULTS AND DISCUSSION

- **Lipopolysaccharide Profiling of ESBL producing *Proteus* isolates in raw milk and milk products**

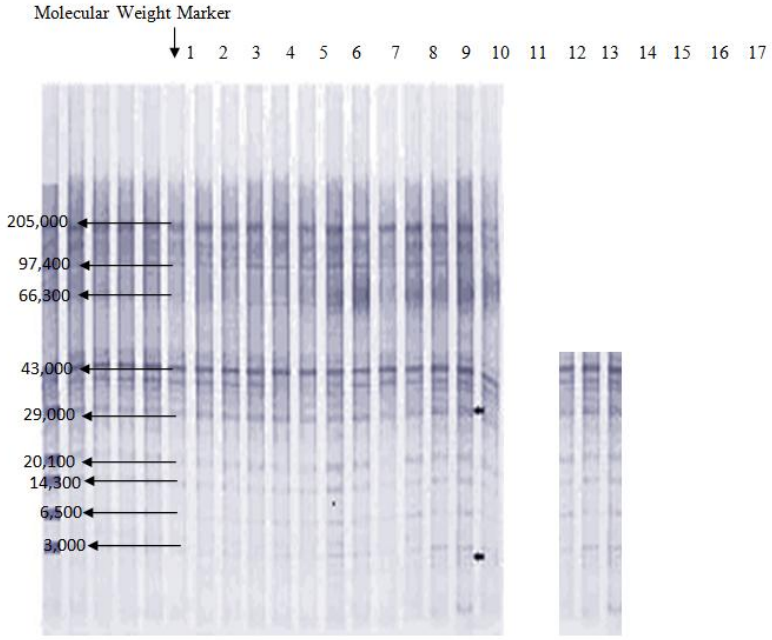

Figure 1: SDS-PAGE analysis of LPS of ESBL producing *Proteus* isolates in raw milk and milk products

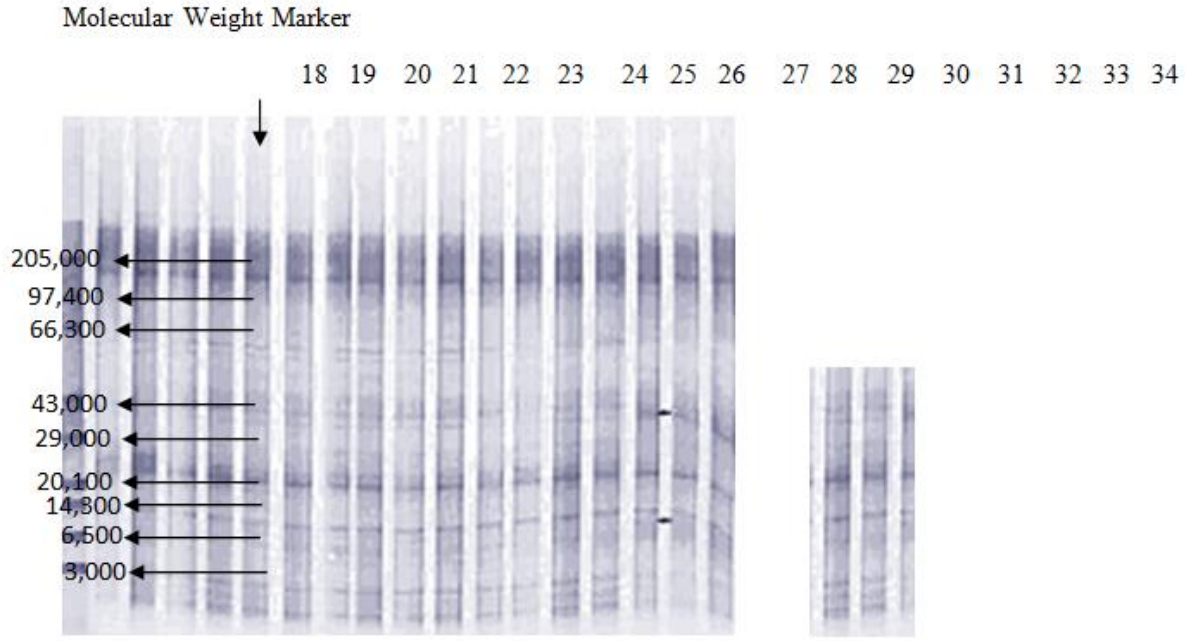

Figure 2: SDS-PAGE analysis of LPS of ESBL producing *Proteus* isolates in raw milk and milk products

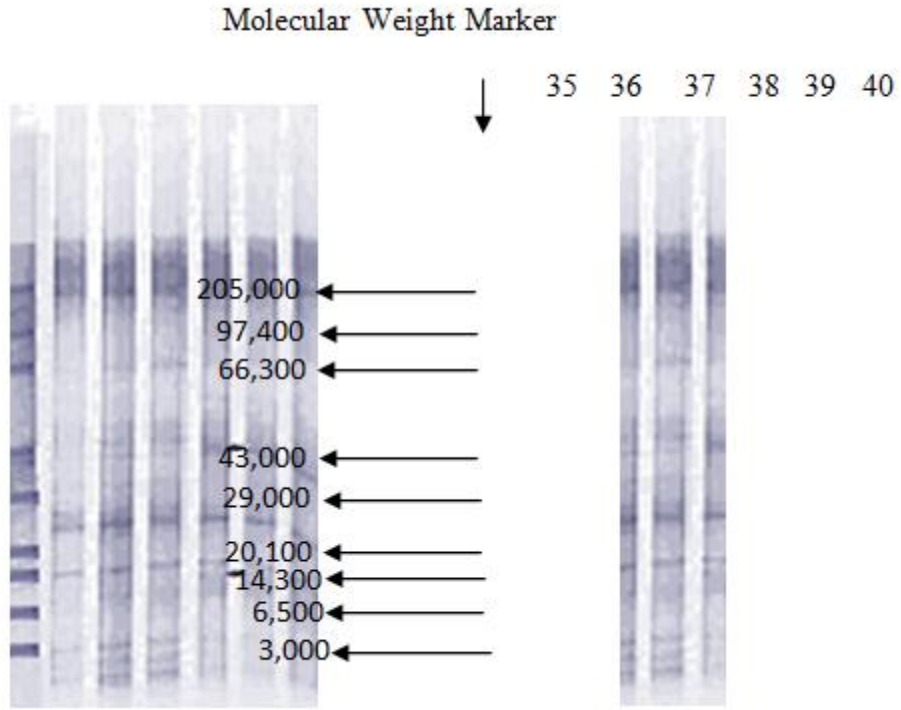

Figure 3: SDS-PAGE analysis of LPS of ESBL producing *Proteus* isolates in raw milk and milk products

The LPS of different strain produced dark "stair case" pattern of bands due to carbohydrate chain length variation of O-antigen portion. The silver staining method for LPS was also standardizing by using different concentration of LPS. The observed band pattern was found to be as such as band pattern reported by Tsai and Frasch (1982). Each band pattern represents a molecule of LPS with increasing unit of O-polysaccharide. Mainly two types of band pattern were observed. Some intense and highly concentrated bands were observed at upper gel layer and some intense bands which were not clearly distinguished were observed at lower surface. The bands at bottom were supposed to have short chain O-polysaccharide and those at the top have long chain of O-polysaccharide. The band patterns of all the isolated strains were observed .60% of the isolates show the band pattern of LPS with some upper intense and lower intense bands. Some bands supposed to having less chain of O-polysaccharide and some are supposed to be high chain of O-polysaccharide. The Silver staining result and intensity of band show isolation of high amount of LPS and also indicate the LPS level in all the strains. The LPS pattern of 60% of the isolates show the band pattern of LPS with intense and highly concentrated bands so these isolates have long chain O- polysaccharides due to dark "stair case" pattern of bands , were observed at the top of the gel. These isolates require high amount of antibiotics concentration for killing. Only those (ESBL producing *Proteus)* isolates which have higher value of MIC give the dark "stair case" pattern of bands at the top of the gel. So these isolates cause more pathogenicity or these ESBL producing *Proteus* isolates were more pathogenic.

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