AJPS, 2012, Vol. 11, No.1

Respiratory Distress and the Bacteria Causing Infection in the Neonates

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الخلاصة:

حللت 64 عينة من عينات (الدم، المهبل والمخرج) للامهات الحوامل واطفالهم حديثي الولادة، اظهرت 58 منها وجود الانواع البكتيرية *Streptococcus agalactiae Staphylococcus* (GBS) *aureus*, *Escherichia coli* ، وكانت عزلات البكتريا موزعة كالاتي:16عزلة من دم الامهات و 25 عزلة من دم حديثي الولادة، بينما 13عزلة من عينات المهبل للامهات و 4 عزلات من عينات المخرج لحديثي الولادة.

نقيت المكونات البكتيرية (الدهون الفوسفاتية PL) بواسطة الـ High Performance Liquid Chromatography (HPLC) وشخصت على انها Cardiolipin (CL), بوقت الاحتجاز (10.76 دقيقة) Phosphatidylserine (PS)، (8.1 دقيقة)، Phosphatidylethanolamine (PE) (5.9 دقيقة) مقارنة بالـ PL القياسي.

اختلفت الانواع البكترية الثلاثة في مستوى ضراوتها عند حقنها داخل القصبات الهوائية لصغار الارانب المختبرية لتعيين الاصابة الرئوية فيها. بعد 8 ايام كانت معدلات الارانب الحية 2/15 (13.33%) لـ GBS ، 5/15 (33.33%) لـ *E.coli*, 7/15 (46.66%) لـ *S.aureus*.

ان بيان علاقة افراز الدهون الفوسفاتية البكتيري وكونه المسبب للضيق التنفسي في حديثي الولادة نتيجة اصابات هذه الانواع البكتيرية قد يفتح افاق واسعة وجديدة للطرائق العلاجية المتعلقة بذلك.

Abstract:

An analysis of 64 samples of (blood, vagina, anaus) from pregnant mothers and their neonates, only 58 samples showed these types of bacteria *Streptococcus agalactiae*(GBS), *Staphylococcus aureus* and *Escherichia coli*, distributed as: 16 blood isolates from mothers and 25 from neonates, while 13 vaginal isolates from mothers and 4 anal isolates from neonates.

Bacterial components (phospholipids-PL) purified by High Performance Liquid Chromatography (HPLC), and identified as Cardiolipin (CL) with retention time (10.76 min.), Phosphatidylserine (PS) (8.1min.), and Phosphatidyl ethanolamine (PE) (5.9 min.) compared with standard PL. GBS isolates produced more extracellular phospholipids than other types in this study.

The three types of bacteria differed in their levels of virulence when injected intratracheally in to a neonatal rabbit model to determine whether they induced pulmonary hypertension in it. After 8 days the rates of surviving neonatal rabbits were 2/15 (13.33%) to GBS, 5/15 to *E.coli* (33.33%), and 7/15 (46.66%) to *S.aureus*. The recognition that bacterial phospholipids may cause respiratory distress in newborns with these kinds of bacteria opens new avenues for therapeutic intervention.
Key words: Respiratory Distress, *Streptococcus agalactiae*, *E.coli*, Neonates *Staphylococcus aureus*.

Introduction:

Group B Streptococci, *Staphylococcus aureus* and *Escherichia coli* are responsible for most cases of sepsis and meningitis in the human newborn [1]. Neonates with these bacteria infections are often quite ill and fatality rate is 5%[2]. Vertical transmission of GBS, *S.aureus and E.coli* from mother to infants can occur after ascending infection of the placental membranes contaminated vaginal fluids during delivery [3].

Respiratory distress, a prominent sign in these babies, is caused by pulmonary hypertension induced by GBS, *S.aureus and E.coli*. The pulmonary hypertension reflects an increase in pulmonary vascular resistance, which impairs exchange of O_2 and CO_2 [1]. The infection leads to pulmonary inflammation with accumulation of cytokines in the lung, leakage of plasma proteins to the alveoli and surfactant inactivation [4].

Respiratory symptoms in the first several hours of life, reflects an initial pulmonary focus of infection. Pathologic changes in neonatal pneumonia include: bacterial and neutrophilic infiltrate, intra-alveolar edema, hyaline membranes, focal atelectasis and evidence of pulmonary epithelial and endothelial cell infiltrates. The bacterium penetrates lung barriers to reach the blood stream, which frequently results in complications such as septicemia and meningitis [3]. Complex mixture of phospholipids probably plays a key role in the patho-physiology of acute respiratory distress syndrome (RDS) due to pneumonia in neonates, infants, and adults. Distruption of the epithelial-endothelial barrier leads to leakage of plasma proteins in to the airspaces [1].

The purpose of this study is to evaluate whether babies are more at risk of developing breathing problems when their mothers carry the bacteria GBS, *S.aureus and E.coli* in vagina/rectum, and whether the breathing problems is due to some components of bacteria like phospholipids, this also accomplished using neonatal rabbits.

Materials and Methods:

Sixty four women (at 32 or more weeks of pregnancy) who deliver at AL-Elwiya- maternity hospital in Baghdad, and their neonates (the period from March/2010 to September/2010), who suspected respiratory distress syndrome

(RDS) with any clinical signs (chest recession, trychypnoea, fever, gastro-esophageal reflux, bronchiectasis, and unexplained chronic cough). GBS, *S.aureus and E.coli* were isolated from the lower vagina of mothers (upon admission to labor and delivery), and from anal isolates of newborns (during the first 6 days of life) by using a cotton swabs [5], samples of the whole-blood were obtained according to [6]. Swabs were transported in Stuart transport medium to the laboratory [7].

All bacteria of this study identified according to [4,8], and confirmed by api20Strep and api20E.(Bio-merieux/France) to *GBS and E.coli* respectively [9], while working cultures were made according to method reported by Song *et.al.*1996 [8]. Measurment of bacteremia were done according to [10], with CFU/ml calculated by spreading 1ml of whole blood on blood agar plates(Oxoid, England), then incubated for 18-24 hr. at 37ºc in anaerobic conditions to GBS type III (by used 5%CO_2 incubator), and at aerobic conditions to others [9].

Extraction and partial purification of phospholipids from bacteria:

GBS, *S.aureus and E.coli* were grown to mid or late-log phase [1]**.** The precipitated cells were washed with sterile normal saline, heat killed by incubation in a 60ºc water bath for 90min, pelleted by centrifugation and resuspend in saline to an O.D of 2.0 at 650 nm. Heat killed bacteria (3g wet weight) extracted with 40ml of (2:1,v/v) chloroform/ methanol by stirring in flask at 4ºc for 5-6 days. The organic solvent was removed by centrifugation and the resulting pellet was redissolved in methanol [11]. After that, the solvent was evaporated and the methylated lipids were redissolved in 0.2ml of chloroform .

Analysis of phospholipids by High Performance Liquid Chromatography (HPLC):

The extracted solution was applied to (HPLC) (model-1100- at Ministry of Science and Technology) with methanol running at 0.5 ml/min at room temperature. The identity of each peak was confirmed from the retention time of each corresponding lipid reference standard phospholipids peaks, which includes: (Cardiolipin - CL, Phosphatidylethanolamine - PE, and Phosphatidyl serine -PS) (Sigma Chemical Co.) [12,13], but other kinds of phospholipids were not determined because no commercial standard was available to our knowledge.

Animals:

Sixty Pregnant white rabbit (obtained from Baghdad university, college of Science, department of biology), were allowed to deliver normally. (0.025-0.106) kg weight, divided in to four groups, three test group (for each bacteria) and control group (n=15), skin of pup was sterilized with isopropyl alcohol, and 10^7 cfu of freshly prepared suspension of each bacteria (GBS, *E.coli*, *S.aureus*) in 5ml PBS/kg was injected precutaneously in to the trachea, control animals received PBS only. Treated rabbits were immediately allowed to recover and observed for respiratory function, color, signs of distress and any signs that

appeared [1,3]. The survival of the pups were monitored twice daily for 8 days, 1.5ml of blood Samples were obtained from each rabbit for quantitative culture and determination of phospholipids [9,10].

Statistical analysis:

Statistical significant was assessed by using least significant differences-LSD (T-test), P-value < 0.05 was considered significant[10]. Results were expressed as mean ± S.D. all the statistical analysis were done by using Pentium- 4 computer through the (SPSS) program (version-10) and excel application [10].

Results:

Isolation of bacteria: Among 64 samples tested, only 58 isolates were studied, 29 isolate from mothers and 29 from neonates (Table-1), 16 isolates of blood samples from mothers showed 9(56.25%) GBS, 4(25.00%) *E.coli* and 3(18.75%) *S.aureus*, while 25 isolates of blood samples from neonates showed 15 isolates (60.00%) of GBS, 7 (28.00%) *E.coli* and 3 (12.00%) *S.aureus*. Also 13 isolates of vaginal samples from mothers showed 7(53.84%) GBS, 3(23.07%) *E.coli* and *S.aureus* respectively, while only 4 isolates of anus samples from neonates showed 2(50.00%) GBS, 1(25.00%) *E.coli* and 1(25.00%) *S.aureus*, respectively. Results showed significant increased ($p<0.05$) in the number of isolated bacteria from mothers and their neonates, and it differ according to the source of isolation.

Samples	**No. of GBS (%)**	**No. of *E.coli* (%)**	**No. of *S.aureus* (%)**	**Total**
Blood Mothers	9 (56.25) *±0.814	4 (25.00) ±0.506	3 (18.75) ±0.476	16
vagina	7 (53.84) ±0.788	3 (23.07) ±0.476	3 (23.07) ±0.476	13
Blood Neonatal	15 (60.00 ±0.961	7 (28.00) ±O.736	3 (12.00) ±0.476	25
anus	2 (50.00) ±0.351	1 (25.00) ±0.081	1 (25.00) ±0.081	4

Table -1: Comparison of different types of bacteria isolated from different sites of mothers and their neonates.

*significant ($P<0.05$)

Purification and identification of the active components:

The chromatogram of standard shows three peaks of phospholipids (CL, PS, and PE) on HPLC (Fig.1), they considered to be associted with retention time (10.76 min.) to CL., (8.1 min.) to PS and (5.9 min.) to PE.

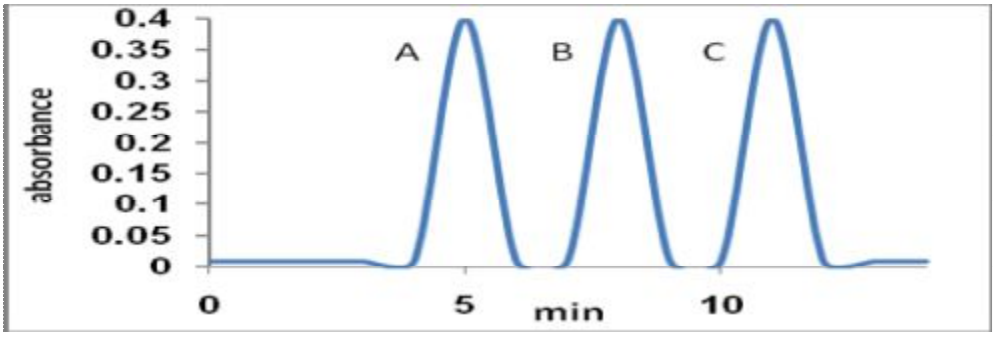

Figure-1: HPLC chromatogram shows standard PL {A-Phosphatidylserine (PS), B-Phosphatidylethanolamine (PE), C-Cardiolipin (CL)}.

Compared to standard phospholipids peaks on HPLC, (Fig.2) shows chromatogram of PL from GBS which exhibited three peaks of CL, PS.and PE. with highest absorbance than others (0.36, 0.18, 0.11) respectively, while (Fig.3) shows chromatogram of PL from *E.coli* which exhibited three peaks of CL, PS.and PE.with absorbance (0.28, 0.12, 0.08) respectively, (Fig.4) shows chromatogram of PL from *S.aureus* which exhibited three peaks of it with absorbance (0.20, 0.10, 0.05) respectively.

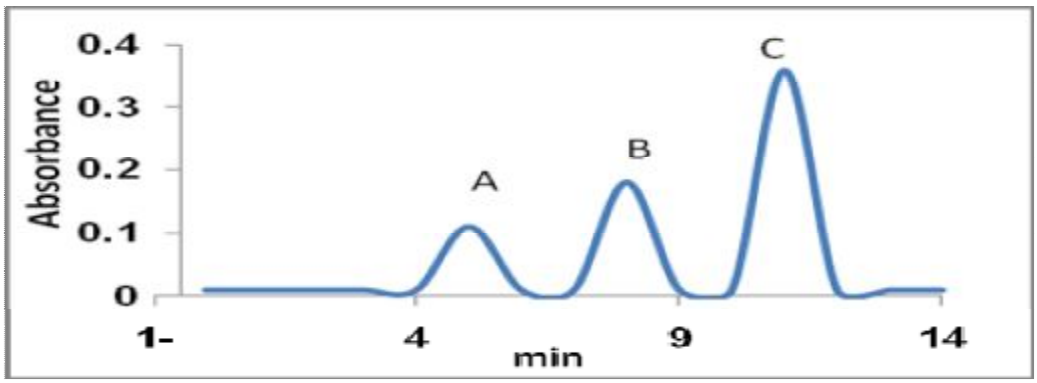

Figure-2: HPLC chromatogram shows PL of GBS isolated from neonatal Blood samples.

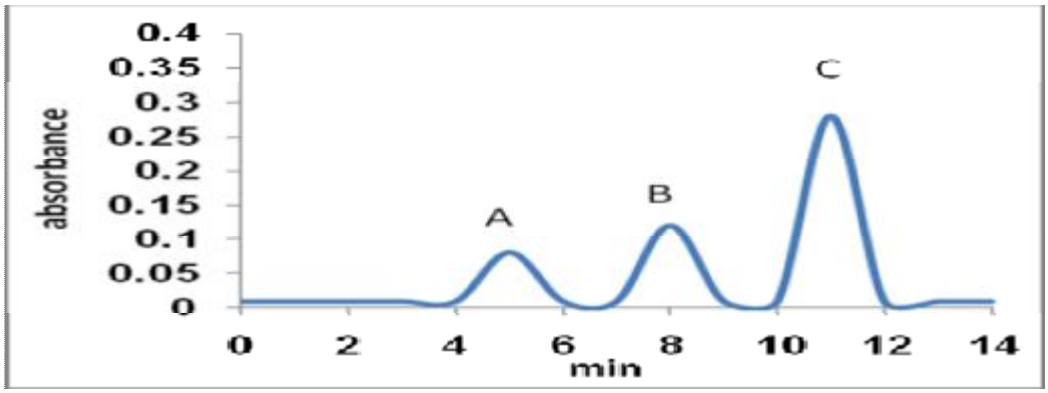

Figure-3: HPLC chromatogram shows *PL* of *E.coli* isolated from neonatal Blood samples.

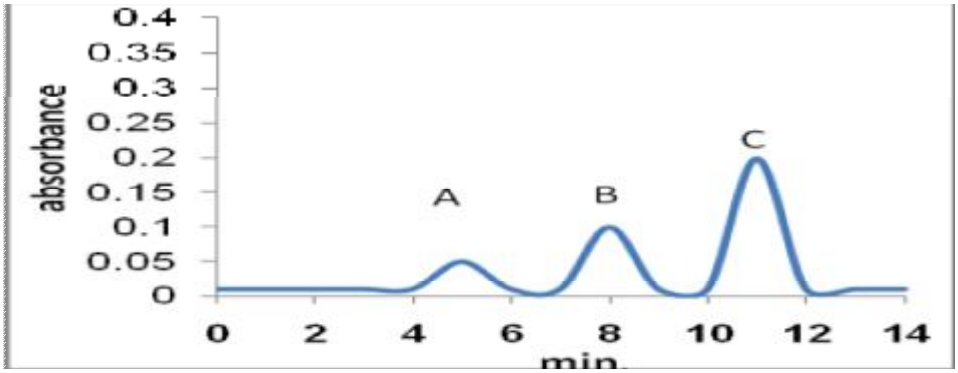

Figure-4: HPLC chromatogram shows PL of *S. aureus* isolated from neonatal Blood samples.

Bacteremia and respiratory distress in rabbits:

Within 24hr. of injection, several pups of rabbits infected with bacteria showed trachypnea and labored breathing. The rate of surviving rabbits after 8 days were 2/15 (13.33%) of GBS, 5/15 of *E.coli* (33.33%), 7/15 of *S.aureus* (46.66%) (Fig.5), and 13/15 (86.66%), 10/15 (66.66%), 8/15 (53.33%) had died with GBS, *E.coli and S.aureus*, respectively. The remainder of the treated group exhibited obvious cyanosis, lethargy, and respiratory distress. In contrast, no rabbits of control group died, and all were normal ain appearance, with no respiratory symptoms after 8 days after injection.

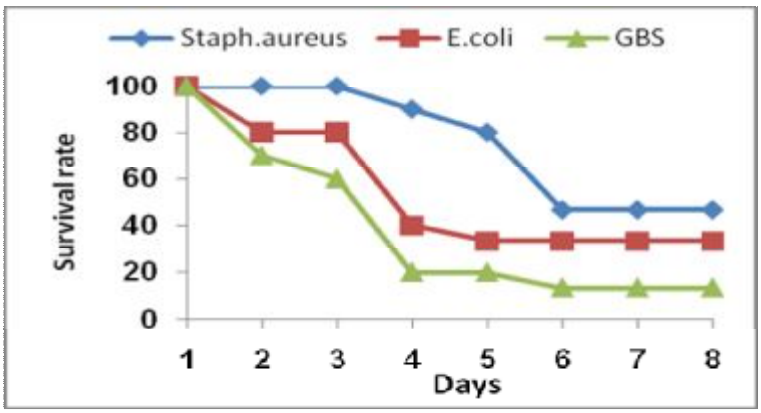

Figure-5: Survival of rabbits injected intratracheally with 10^7 CFU/ml bacteria of of GBS-▲, *E.coli*-■, *S.aureus*-♦

Positive blood culture of *E.coli*, *S.aureus* and GBS were demonstrated after each treatment, in the first hour of injection, bacteremia was at a level of 10^7 CFU/ml blood for all types of bacteria including the study, after 2 hours of injection , bacteremia was at a level of 10^6 CFU / ml blood for GBS and *E.coli* respectively, 10^5 for *S.aureus*. Bacteremia declined to 10^5 CFU/ml after 5hr. injection by GBS, 10^4 for *E.coli*, and 10^3 for *S.aureus* (Fig.6).

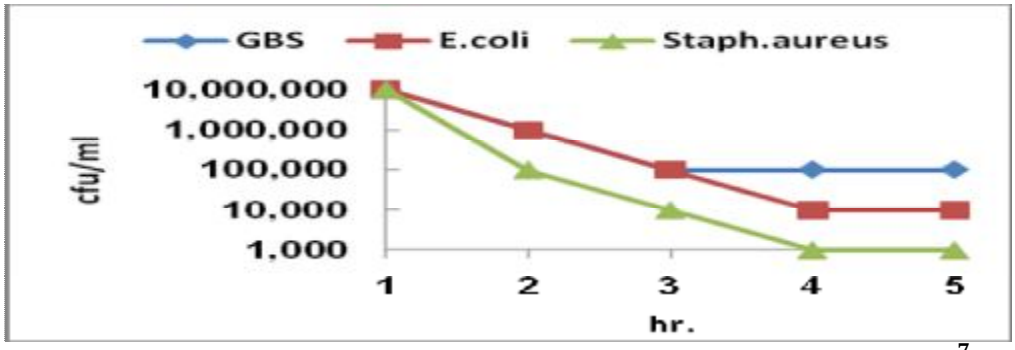

Figure-6: Bacteremia in rabbits injected intratracheally with 10^7 CFU/ml of GBS-♦, *E.coli*-■, *S.aureus*-▲

These results demonstrate that phospholipids contributes to GBS, *S.aureus* and *E.coli* proliferation in lung tissues, promotes penetration of the pulmonary and epithelial barriers, and produces high lethality in the neonatal infection.The key finding in our study is that bacterial phospholipids cause pulmonary distress. Identification of the receptors and pathways that are activated by these phospholipids may allow discovery of therapeutic interventions, and experimental animals may help the future studies to examine the pathophysiology of neonatal pneumonia.

Discussion:

Physiologic conditions vary considerably between the rectum, vagina, and lung [2], and the surface culture of babies excluding umbilical swab like external auditory canal, anal and gastric aspirate culture correlated with blood culture, and there is correlation between surface culture and blood culture of babies [10]. Valentin-Weigand, *et.al* 1995 [14] showed that among 77 blood isolates tested from invasive infections of neonates, 48 isolates of GBS caused respiratory signs in them, and its more invasive than other sites, while Alarcon,*et.al* 2004 [15] exhibited that 41 of 58 live births having signs of respiratory distress caused by *E.coli* infection. Steps considered to be important in the pathogenesis of neonatal infections include colonization of the rectum and vagina of the mother, aspiration of bacteria into the fetal lung during or just prior to delivery, and invation of it in to pulmonary epithelial cells [16].

GBS, *S.aureus and E.coli* is an important cause of maternal and neonatal morbidity and mortality in many parts of the world [17, 18, 19]. Bouhafs and Jarstrand, 1999 [20] showed that three bacterial species, GBS, *E.coli*, and *Pseudomonas aeruginosa* invade the epithelium of lung and cause lipid peroxidation. The infant respiratory distress syndrome caused by different strains of GBS, *S.aureus, S.epidermidis* and *E.coli* which are important neonatal pathogens [21]. Prevention of the infection can be determined by identifying and treating pregnant women who carry GBS, *S.aureus*, and *E.coli* or who are at highest risk of transmitting the organisms to newborn. The intrapartum use of

antibiotics in these women has led unequivocally to a decrease in the rate of neonatal disease [18,19].

Phospholipids are the major components of bacterial cell membranes, these lipid compounds posses closely related and complex structures classed according to the polar head group linked to the phosphate moiety. The diversity of the polar head groups is important, and as far as prokaryotic organisms are concerned, the main classes of phospholipids are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and their respective derivatives [22]. CL is an anionic phospholipid present in mitochondrial and bacterial membranes, and generally is not recognized as a significant physiologic plasma component. CL was considered not to be exposed to circulating antibodies and, therefore, not likely among the phospholipids to be an autoantigen; PS, also found in lipoprotein [6]. Rauprich, *et.al* 2000 [18] compared the influence of phospholipids on different reference strains and several clinical isolates of GBS, *S.aureus*, and *E.coli, w*hile Phosphatidylglycerol and cardiolipin are the dominant phospholipids of GBS, located mainly in the cell wall [1].

Several methods were established for purifying phospholipids , one of them was HPTLC blotting which is simple and specific [23,24,25]. The total phospholipids from children with cystic fibrosis was achieved by HPLC analysis were PC, PG, and PI classes [26], also crude phospholipids obtained from *Strep. thermophilus* was physicochemically and biochemically characterized as a multicomponent biosurfactant, and fractionated using hydro-phobic interaction chromatography [27].

The three active fractions established that they were phosphatidylserine (PS), phosphatidyl-ethanolamine (PE) and cardiolipin (CL), each caused pulmonary distress in the rabbit assay [9]. The observation in this study showed that GBS challenge of rabbits is associated with increased mortality, compared with other types of bacteria, induced substantially higher mortality without an apparent advantage in establishing and maintaining bacteremia. Significant bacterial proliferation associated with septicaemia could be induced in more than 80% of the infected animals [27].

Lewis, *et.al*, 2004[28] showed that instillation of GBS *and E.coli* in rats intratraceal increased mortality caused by bronchopneumoniam compared with controls. Phospholipids plays a critical role in GBS-induced pulmonary dysfunction and in the ability of the pathogen to penetrate the lung barrier, establish bateremia, and produce high mortality, and after 2-3 days 31 of the infected animals by GBS showed symptoms of respiratory failure [3, 10]. Sheman, *et.al*, 1988 [29] showed that newborn rabbits received intratracheally artificial phospholipids followed by pulmonary infection with GBS after four hours of lung infection.

These results demonstrate that phospholipids contributes to GBS, *S.aureus* and *E.coli* proliferation in lung tissues, promotes penetration of the pulmonary and epithelial barriers, and produces high lethality in the neonatal

infection.The key finding in our study is that bacterial phospholipids cause pulmonary distress. Identification of the receptors and pathways that are activated by these phospholipids may allow discovery of therapeutic interventions, and experimental animals may help the future studies to examine the pathophysiology of neonatal pneumonia.

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