

## THE PATHOLOGICAL EFFECT OF PEPTIDOGLYCAN ON RATS' LUNGS PART ONE: PATHOGENIC BACTERIA *STREPTOCOCCUS PNEUMONIAE*

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### Abstract

Twenty isolates of *Streptococcus* were obtained from fifty sputum specimens collected from patients presented with respiratory tract infections. Two of these isolates were identified as *S. pneumoniae* by conventional biochemical tests. For extraction of peptidoglycan from *S. pneumoniae*, mechanical disintegration by glass beads and vortex plus enzymatic digestion by DNase, RNase and pronase were applied. The partial purified peptidoglycan showed four protein bands compared with crude peptidoglycan which showed six bands when performed in polyacrylamide gel electrophoresis under undenaturing conditions.

The rats injected with germ suspension or the peptidoglycan showed similar histopathological changes included disruption of alveoli walls, haemorrhage, infiltration of inflammatory cells, sloughing of the epithelial cells lining the bronchioles, and edema. The effects of histopathological changes of the peptidoglycan of *S. pneumoniae* were more severe than those of cell suspension.

### Introduction

*Streptococcus pneumoniae* is a leading cause of pneumonia in all ages often after "damage" to the upper respiratory tract (e.g. following viral infection). It also causes middle ear infections (otitis media). The organism often spreads causing bacteremia and meningitis. *S. pneumoniae* is  $\alpha$  hemolytic and there is no group antigen. Direct Gram staining or detection of capsular antigen in sputum can be diagnostic. The organism grows well on sheep blood agar (1,2).

Although the structure varies in gram positive and gram negative cells, the rigidity of bacterial cell walls is due to a layer of peptidoglycan, a macromolecule found only in bacteria. The basic structure of peptidoglycan is an alternating series of two major subunits, N- acetylmuramic (NAM) acid and N-acetylglucosamine (NAG). These subunits, are covalently joined together to form a glycan chain which serves as the backbone of the peptidoglycan molecule. Attached to each of the NAM molecules a string of four amino acids; a tetrapeptide chain. Cross-linkage can form between tetrapeptide chains, thus joining adjacent glycan chains to form a single, very large three dimensional molecule (2).

In *S. pneumoniae* the peptidoglycan consists of the basic structure, the backbone attached to a tetrapeptide consists of L-alanine,

D-isoglutamic acid, L-lysine and D-alanine (3,4).

Inflammation and septic shock are considered as the characteristic of infection caused by gram positive and gram negative bacteria (5). Recently a great attention was paid to the role of gram positive bacteria in the pathogenicity especially septic shock (6,7).

It was shown that the peptidoglycan, teichoic acid and lipoteichoic acid are immunostimulators as they stimulate the release of tumor necrosis factor (TNF) , IL-1 $\beta$  and IL-6 from Peripheral Blood Mononuclear Cells (PBMCs) (8). However the synergistic effect of peptidoglycan and lipoteichoic acid induced the production of nitric oxide a vasodilator can lead to circulatory failure, hypotension, and vascular hyporeactivity (9).

Injection of peptidoglycan in rats (10) and rabbits (11) was found to cause multiorgan dysfunction due to increase in level of aspartate aminotransferase, alanine aminotransferase and bilirubin which means a hepatic injury has occurred also in level of urea and creatinine and indication of renal dysfunction.

Majcherzyk and his coworkers (12) pointed out to the role of oligomeric stem peptides of *S. pneumoniae* peptidoglycan, but not the mono-peptides or dipeptides, in

inflammation and release the TNF from PBMCs.

The present study aimed to study the pathological effects that may result from *S. pneumoniae* peptidoglycan injection in rats' lungs.

## Materials and Methods

### Isolation

Fifty sputum specimens were collected from respiratory tract infection patients with different age visiting Medical city and central children hospital from November, 1<sup>st</sup> 2004 to March, 1<sup>st</sup> 2005 in order to isolate *S. pneumoniae*.

The specimens were cultured on blood agar and heated blood agar plates at 37° C for 24 h, thereafter, the discrete colonies that produce  $\alpha$  hemolysis were selected for further conventional biochemical tests (13,14).

### Antibiotic sensitivity

Susceptibility to the antibiotics listed in Table 1 is tested using the disk diffusion method described by (15).

**Table (1)**  
**Antibiotics used in study.**

Antibiotic	Disc code	Concentration (µg/ml)
Amoxicillin	Ax	10
Ampicillin	AM	10
Augmentin	AC	20
Ceftzidime	CA	30
Cephalothin	CK	30
Ticarcillin	TC	75
Tobramycin	TM	10

### Peptidoglycan extraction

The peptidoglycan of *S. pneumoniae* was extracted according to Majcherczyk *et al.* (12).

Two liters of brain heart infusion were inoculated with *S. pneumoniae* at 37 °C for 18 h. The bacterial culture was quickly chilled in an ice / ethanol bath at 0 - 4° C. and the cells were harvested by centrifugation at 8000 xg for 20 min at 4 °C. and washed three times by sterile distilled water (DW). Seven milliliters 4% sodium dodecyl sulfate (SDS) were added to the pellets and incubated in boiling water

bath at 100 °C for 15 min. Once again the cells were washed 10 times by sterile distilled water. The precipitate was suspended in 15 ml of DW. Protein (16), carbohydrate (17) and nucleic acids (18) were assayed. Protein electrophoresis was carried out to testify the purity of the extract by the procedure suggested by Piljac and his colleagues (19).

Glass beads of 0.2 mm were added to the suspension and mixed by vortex for 10 min. The suspension was aspirated by aid of Pasteur pipette and centrifuged at 8000 xg for 20 min at 4 °C. The residual was resuspended in 2 ml of 0.1 M Tris-HCl buffer pH 7.5, then treated with DNase (50 µg/ml) and RNase (50 µg/ml) for 2 h. and trypsin (100 µg/ml) for 18 h. The suspension was centrifuged at 3000 xg for 20 min at 20° C. The precipitate was mixed with 3 ml of 1% SDS and incubated in boiling water bath at 100 °C for 15 min. Thereafter, it was washed twice with sterile distilled water. Five milliliters of 8 M Lithium chloride were added to the precipitate of last wash and incubated at 37 °C for 15 min. subsequently; five milliliters of 100 mM EDTA were added. The suspension was centrifuged at 3000 xg for 15 min at 4 °C. and the residual was washed with DW. Ten milliliters of acetone were added to the precipitate and submitted to dryness at room temperature. Dry weight was estimated in addition to protein (16), carbohydrate (17) and nucleic acids (18). Protein electrophoresis was carried out to testify the purity of the extract (19).

### In vivo study

#### Animals

White female rats, aged 6–7 weeks, weighing 260 to 330 g were obtained from the animal house of University of Baghdad, College of Science, Department of biology. The animals were divided into three groups (A, B and C) as three animals per group.

#### Injection protocol

Group A was injected intranasally using sterile catheter (0.6 mm in diameter) with the *S. pneumoniae* peptidoglycan ( $37 \times 10^4$  µg/ml). Group B was injected with  $1 \times 10^9$  cfu / ml of bacterial suspension while group C was injected with normal saline following the same manner achieved with group A.

Two days later, the animals were killed and the lungs were preserved in 10 % formalin as they prepared for histopathological study (20).

### Results and Discussion

Out of 20 *Streptococcus* isolates, 2 (10 %) isolates were identified as *S. pneumoniae*. A result is considered very low in comparison to other studies. Soepandi and his colleagues (21) isolated 47 bacterial isolates from 34 sputum specimens collected from patients presented with acute pulmonary infections, *S. pneumoniae* isolation percentage reached 10.63 %. And in another study it reached 25.8 % (22), while Alqaddo (23) and Ibrahim (24) have isolated the pneumococci separately in percentage reached 24 % and 34.8 % respectively.

The two isolates were susceptible to tobramycin, augmentin and ampicillin.

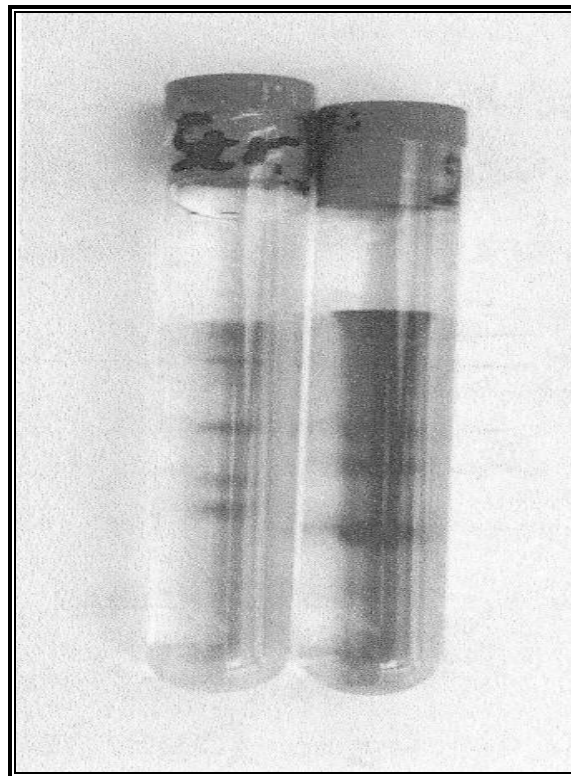
Table (2) demonstrated the result of protein, carbohydrate and nucleic acids estimation. The protein concentration of the peptidoglycan extract dropped from 150  $\mu\text{g} / \text{ml}$ , at the first step of extraction after treatment with SDS, to 21.25  $\mu\text{g} / \text{ml}$  at the end of extraction process, an indication of the efficiency of the procedure of extraction. However, carbohydrate concentration increased from 24 to 91  $\mu\text{g} / \text{ml}$ . nucleic acids estimation was accomplished as the RNA and DNA were dropped from 1.2 and 1.0, respectively, to 0.0  $\mu\text{g} / \text{ml}$ . These results agreed with Umeda *et al.* (25) and Al-heety (11).

**Table (2)**

**Protein, carbohydrate and nucleic acids estimation of the peptidoglycan extracted from *S. pneumoniae*.**

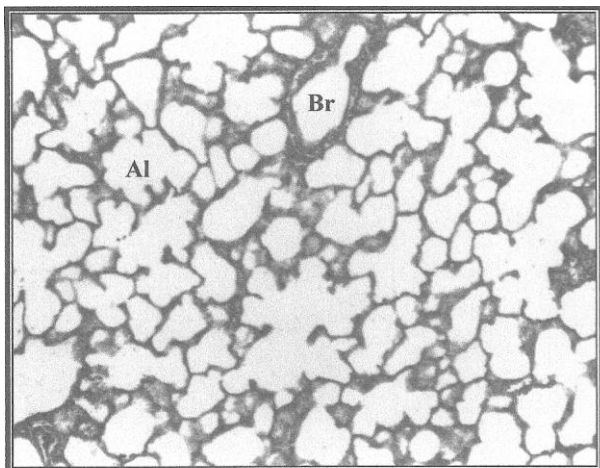
Extraction step	Protein concentration $\mu\text{g}/\text{ml}$	Carbohydrate concentration $\mu\text{g}/\text{ml}$	Nucleic acids concentration $\mu\text{g}/\text{ml}$	
			DNA	RNA
After treatment with 4% SDS	150	24	1.0	1.2
Last step of the extraction	21.25	91	0.0	0.0

Fig. (1) illustrated the result of electrophoresis to indicate the purity of peptidoglycan extract, as we can see the specimen has developed six bands after treatment with SDS while they were reduced to 4 bands at the end of extraction procedure.

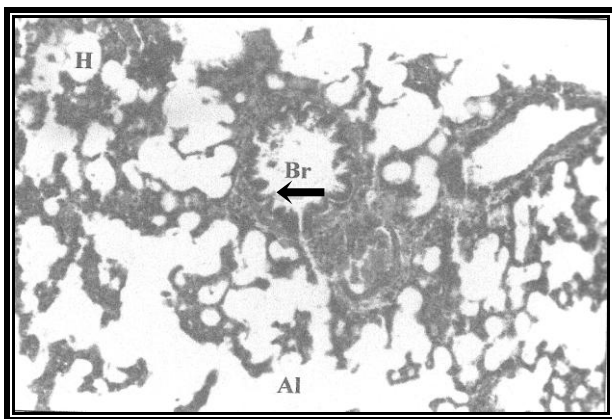


**Fig.(1) : Electrophoresis of *S. pneumoniae* peptidoglycan after treatment with SDS (right) and at the end of extraction procedure (left).**

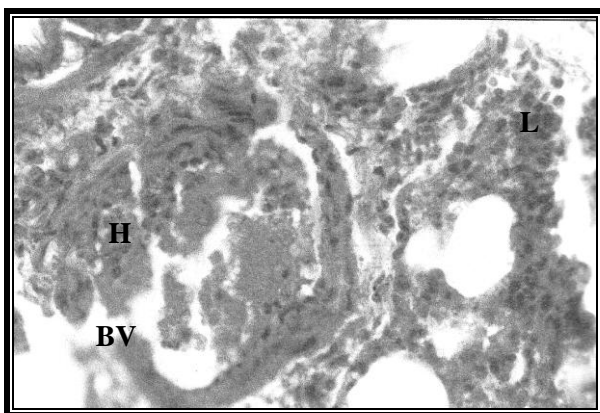
The results of the histopathological study of the rats' lung injected with *S. pneumoniae* showed several pathological changes in comparison to control Fig.(2) represented by disruption in the alveoli walls, haemorrhage, as a body response to these foreign bodies, sloughing of epithelial cells inside the bronchiole and hydropic degeneration of cells lining the bronchiole Fig.(3) disruption in the vessel wall and infiltration of inflammatory cells Fig.(4).



**Fig. (2) :** Cross section in rat lung showing the alveoli (A1) and the bronchioles (Br). X400 H&E.

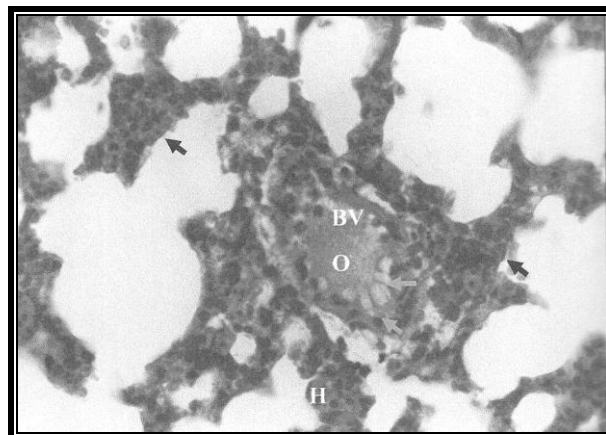


**Fig.(3):** Cross section in rat lung injected with  $1 \times 10^9$  cfu / ml of *S. pneumoniae* showing disruption in the alveoli walls (A1), haemorrhage (H), sloughing of epithelial cells inside the bronchioles (Br) and hydropic degeneration of cells lining the bronchiole (arrow) X400 H&E.



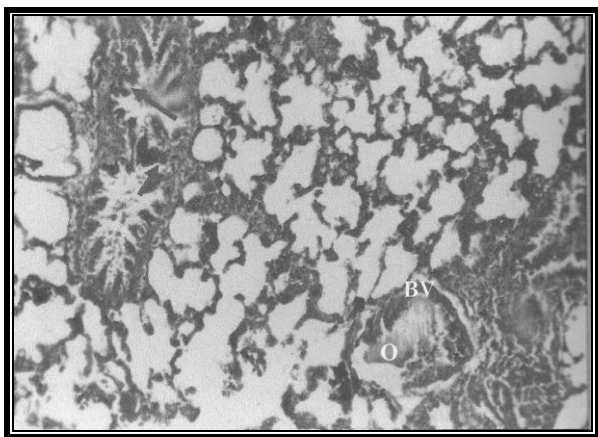
**Fig.(4):** Cross section in rat lung injected with  $1 \times 10^9$  cfu / ml of *S. pneumoniae* showing disruption in the vessel wall (BV), haemorrhage (H), infiltration of inflammatory cells (L) X400 H&E.

Edema have been seen inside the lung tissue due to imbalance of hydrodynamic forces through pulmonary capillaries in addition to increase the permeability of capillary endothelial layer which result in leaking of fluids more than the adjacent tissue, plus the vacuolation inside the blood vessels due to the cellular transport hyperactivity of the endothelial cells Fig.(5) (26).

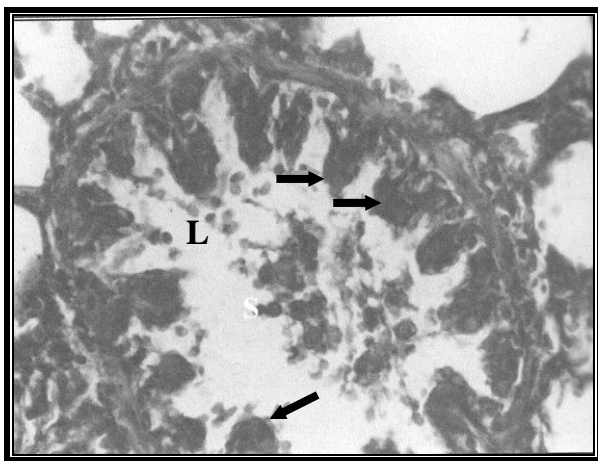


**Fig.(5) :** Cross section in rat lung injected with  $1 \times 10^9$  cfu / ml of *S. pneumoniae* showing edema (O), vacuolation (V), haemorrhage (H) and infiltration of inflammatory cells (arrow). X400 H&E.

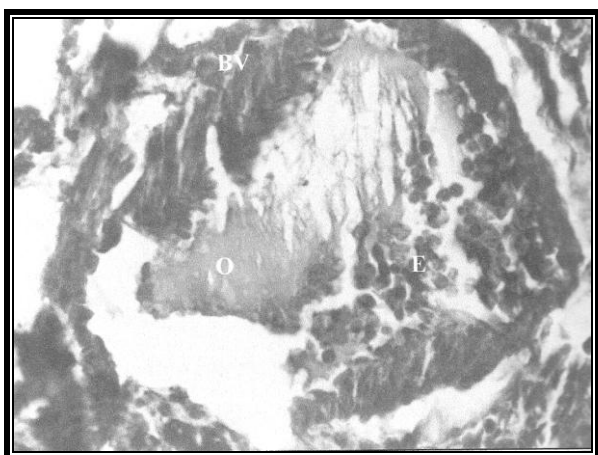
The peptidoglycan caused many pathological changes demonstrated by disruption of alveoli walls, edema inside the blood vessel and hydropic degeneration of cells lining the bronchioles Fig.(6) and sloughing of epithelial cells lining the bronchioles, in addition to aggregation of leukocytes as it illustrated in Figs. (7) and aggregation of erythrocytes (congestion) Fig.(8).



**Fig.(6):** Cross section in rat lung injected with *S. pneumoniae* peptidoglycan ( $37 \times 10^4 \mu\text{g/ml}$ ) showing disruption of alveoli walls (D), edema (O) in blood vessel (BV), hydropic degeneration (arrow). X400 H&E.



**Fig.(7):** Cross section in rat lung injected with *S. pneumoniae* peptidoglycan ( $37 \times 10^4 \mu\text{g/ml}$ ) showing hydropic degeneration (arrow), sloughing of epithelial cells (S) leukocytes (L). X1000 H&E.



**Fig.(8):** Cross section in rat lung injected with *S. pneumoniae* peptidoglycan ( $37 \times 10^4 \mu\text{g/ml}$ ) showing edema (O) and aggregation of erythrocytes (E) in blood vessel (BV),. X1000 H&E.

It is so noticeable that the peptidoglycan effects were more severe than bacterial suspension since the amount of sloughing of the epithelium made by peptidoglycan was more than by bacterial suspension in addition the extent of damage that occurred in the blood vessel represented by amount of infiltration of inflammatory cells, hydropic degeneration and congestion.

Our results indicate that Peptidoglycan of *S. pneumoniae* play an important pathogenic role in inflammatory lung disease.

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## الخلاصة

تم الحصول على عشرين عزلة *Streptococcus* خمسين عينة قشع جمعت من مرضى مصابين بخمج المجاري التنفسية و اثبت عائدة عزلتين منها الى النوع *S. pneumoniae* بوساطة الطرائق الكيماحيوية التقليدية . ولغرض استخلاص الببتيدوكلايكان من هذه البكتريا تم اعتماد التكسير الميكانيكي بوساطة الكرات الزجاجية والمازج مع اله ضم الانزيمي بانزوي مات الدناز والرناز والبروناز . وعند اجراء الرحلان الكهربائي عند ظرف غير ماسخة، اظهر الببتيدوكلايكان المنقى جزئيا اربع حزم بروتينية مقارنة مع الببتيدوكلايكان الخام الذي اظهر ست حزم.

اظهرت الجرذان المحقونة بعالق خلايا *S. pneumoniae* او بالببتيدوكلايكان تغييرات نسيجية مرضية متشابهة شملت تمزق جدران الحويصلات الرئوية و النزف و ارتشاح الخلايا الالتهابية وانسلاخ الخلايا الطلائية المبطنة للقسيبات و تكوين الودمة . في حين كانت التغييرات النسيجية المرضية الناتجة عن الببتيدوكلايكان اشد من تلك الناتجة عن عالق الخلايا.