

## Isolation and Identification of *Legionella pneumophila* From Different Sources<sup>♦</sup>

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### الخلاصة

تضمنت الدراسة عزل وتشخيص جرثومة *Legionella pneumophila* من قشع المرضى الوافدين في مستشفى كوماري ورزكاري باجناس واعمار مختلفة في مدينة اربيل ، بالاضافة الى عزلها من مصادر مائية مختلفة ، اذ جمعت (652) عينة من قشع المرضى و(100) عينة من مياه حنفية وشورات الاستحمام و (72) عينة من برادات المياه. وقد تم الحصول على (20) عزلة للجرثومة وبنسبة (8.33%) من قشع المرضى بالاضافة الى (19) عزلة وبنسبة (19%) من مياه حنفية وشورات الاستحمام و (11) عزلة وبنسبة (15.27%) من برادات المياه.

تم تشخيص عزلات الجرثومة اعتمادا على الصفات الشكلية والمزرعية والكيموحيوية . وعلى اساس تباين العزلات في استجابتها لبعض الاختبارات مثل اختبار البروتينز و DNase وتحلل النشاء وعلى اساس نتائج هذه الاختبارات الثلاث تم تنميط العزلات حيويًا (Biotyping) الى ثمانية انماط حيوية وقد وقعت اغلب العزلات ضمن النمط الحيوي الاول. كما ابدت جميع العزلات تفاعلا موجبا لانتاج انزيمات lipase و lecithinase و  $\beta$ -lactamase.

كما تم التحري عن بعض عوامل الامراضية للجرثومة كقدرتها على الالتصاق على الخلايا الطلائية المبطنة لتجويف الفم ولوحظ ان جميع العزلات لها قدرة الالتصاق على الخلايا الطلائية.

شملت الدراسة ايضا استخلاص وتحليل متعدد السكريات الدهني باعتماد تقنية الهجرة الكهربائية SDS-PAGE وظهرت النتائج وجود تباين في عدد الحزم و اوزانها الجزيئية بين العزلات المرضية والبيئية.

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### **Abstract**

This study includes the isolation and identification of *Legionella pneumophila* from patient's sputum in different ages for both sexes in Komary and Rizgary Teaching Hospital in Erbil city, in addition to aquatic samples from different sources, (652) samples were collected, specimens from patients sputum, (100) water samples from bathroom tap and shower heads and other (72) water samples from cooling devices. From the patients sputum samples, (20) isolates with percentage (8.3%) were obtained, while (19) isolates (19%) from bathroom tap and shower heads and (11) isolates (15.27%) from cooling devices were isolated.

All isolates were identified depending on morphological, cultural, and biochemical characteristics. Depending on the results of some enzymatic activities such as protease, DNase, and starch hydrolysis enzymes, all isolates typed to eight biotypes and most of isolates were located in biotype I . Also all isolates were gave positive reactions for production of lipase, lecithinase, and  $\beta$  -lactamase.

The present study also involves the investigation of its ability to adhere to the epithelial cells of buccal cavity, and it was observed that all of the isolates have the ability to adhere to the epithelial cells.

The study also included the extraction and analysis of lipopolysaccharide (LPS) by using SDS -PAGE electrophoresis technique and the results showed that there were differences in the LPS bands number and molecular weights between clinical and environmental isolates.

### **INTRODUCTION**

Acute respiratory infections (ARI) is one of the most important causes of morbidity and mortality in humans throughout most of the world (1). The genus *Legionella* is one of the most abundant of all aquatic bacteria, consisting of over 50 species, with more than 70 serogroups. Approximately 70 to 90 % of *Legionella* infections are caused by *Legionella pneumophila* and other species are responsible for between 5 to 30 % of the cases of infection (2).

Since *Legionella pneumophila* was first recognized as a disease entity from a pneumonia outbreak in July in 1976, the convention of the American Legion in Philadelphia was struck down with a mysterious acute respiratory illness (3). Investigation of the outbreak by Joseph McDad, in Centers for Disease Control and Prevention (CDC), led to the eventual discovery of the causative agent in January of 1977(7). The bacterium was subsequently named *Legionella pneumophila* and the disease termed legionellosis which is a disease has two distinct forms legionnaires disease and Pontiac fever (4). This bacterium belongs to the family *Legionellaceae*, it is faintly stained gram negative rods,

uncapsulated, non-spore forming, with dimension from (0.3 - 0.9)  $\mu\text{m}$  in width and (2- 20)  $\mu\text{m}$  in length, most exhibit motility by one or more polar or lateral flagella, and its cell wall contains lipopolysaccharide (LPS), which is unique from other gram negative bacteria in that its lipid consists of significant amounts of long branched-chain cellular fatty acid (5).

The primary isolation of *Legionella pneumophila* in laboratory is difficult, because it is fastidious and slow growing as well as it requires prolonged incubation period (3 - 10) days; therefore, culture on Buffered Charcoal Yeast Extract Agar (BCYE) plates was considered the gold standard for the laboratory diagnosis of the bacterium (6).

## Materials and Methods

### Collection of samples:

A total of 412 samples were collected within four months (November 2005 to March 2006) from different sources:-

- (240) sputum specimens were taken from patients in Komary and Rizgary Teaching Hospital in Erbil city, comprising 144 (60%) with an age ranged between 5-75 years old. The specimens collected at first morning in a sterile cup and transferred directly to the laboratory for microbiological examination.
- (172) water samples from different water sources were taken; 100 water samples from the bathroom tap and shower heads in different private homes and 72 other water samples from cooling devices in different places were taken. The water samples were collected in sterile bottles and immediately brought to the laboratory and subjected directly to microbiological examination.

### Culture media for isolation of *L. pneumophila*:-

Various culture media were used to isolate this pathogen and all of these media were supplemented with iron salts and L-cysteine HCl as a growth factors, also all culture media were modified by using ferric nitrate in place of ferric pyrophosphate as a source of iron in concentration 0.25g/L (6).

- 1- Buffered Charcoal Yeast Extract Agar Medium (BCYE): prepared as described by (6).
- 2- Feeley Gorman Agar Medium (FG): prepared as described earlier (7).  
The amino acid tyrosine (0.4 g/L) was added to enhance the growth of *Legionella*.
- 3- Yeast Extract Tyrosine Agar Medium (YET): (8).
- 4- Modified Wadowsky Yee Medium (MWY): (9).

After the samples were transferred directly to the laboratory, the (sputum) was inoculated by sterilize swabs in all above media while,

water samples were pretreated with an HCl -KCl buffer mixture at PH 2.2 for 3-5 minutes and then inoculated by transferring 0.1ml of each sample in the same above media and spread with L-shaped rod, then incubated at 37°C for 3-10 days in 2.5- 5% CO<sub>2</sub> by using candle jar (6).

Morphological test, Microscopic examination and Biochemical tests were done according to 5,6,10,11.

### **Biotyping**

All isolates of *L. pneumophila* were biotyped depending on the different results of some enzymatic reactions such as protease, DNase, and starch hydrolysis enzymes which have role in occurrence of infection.

### **Adherence assay**

The adherence assay by using Geimsa stain was conducted to emphasize the pathogenesis of *L. pneumophila*. According to 12,13,14.

### **Extraction and purification of lipopolysaccharid (LPS)**

The LPS from *L. pneumophila* was isolated depending on (15).

### **Results and Discussion:**

A total of 412 samples were collected from different sources; 20 (8.33%) isolates of *L. pneumophila* were isolated from 240 sputum samples. This result was in agreement with that reported by (16), who demonstrated a total of 74 sputum samples 7(9.5%) specimens yielded *L. pneumophila*. This percentage is relatively low and this may be due to that the isolation of *Legionella* from sputum is more difficult because of the predominance of bacteria of other normal flora (5).

On the other hand, our results showed that the incidence of the infection was more common in elderly men than others and these results were in accordance with that reported by (16).

Moreover, 19 (19%) isolates of *L. pneumophila* were isolated from 100 samples of the bathroom tap and shower and these results were similar to that obtained by (17) but lower than other previous studies. 14,15,16.

The first evidence of the association between potable water from shower and nosocomial legionellosis was reported approximately 20 years ago (18). On account of its tolerance to heat and low nutritional needs *L. pneumophila* is widespread in many water systems and the hot water system is thought to be the most frequent source of cases (16, 18). In many outbreaks hot water systems are the most frequently involved sources of infection (16, 18) and it may be due to water temperature, presence of sediment and metals content, type and age of tank and distribution system in addition to uncontinuously cleaning of bath room.

Concerning water samples of 72 cooling device samples, 11(15.27%) samples yielded *L. pneumophila* isolates as appear in and these results were similar to those reported by (17); this percentage was not higher than that results obtained by (19,20) which they isolated 60%, and 93.7% respectively of *L. pneumophila* in their studies.

A number of laboratory prepared media were used. The results showed that selective media have the best efficiency for isolation and growth of *L. pneumophila*. On BCYE Iridescence grayish-white colonies with cut-glass appeared on BCYE agar after 7 days of incubation at 37°C and 2.5-5% CO<sub>2</sub> (fig. 1) and these results were in accordance with (6,21). This medium is an excellent selective medium for *L. pneumophila* because it was supplemented with amino acid L-cystein and ferric nitrate as source of iron that provides energy and carbon and enhances the growth of bacteria (8). In addition to  $\alpha$ -Ketoglutarate and activated charcoal which support *Legionella* growth by reducing production of hydrogen peroxide (22). Also this medium is supplemented with yeast extract as a source of nitrogen (8).

On FG agar medium small browning colonies was appeared (fig. 2), because the medium contain of amino acid tyrosine which stimulated the growth of *Legionella*.

Brown colonies of *L. pneumophila* were detected after an incubation period at 37°C for 7 days on YET medium as showed in fig. (3). The results of our study were in agreement with that reported by (8).

However, this medium consists of essential growth factors such as L-cystin, tyrosine and ferric nitrate in addition to yeast extract as a source of protein (19).

On MWY medium colonies green iridescence with cut-glass shape as shown in fig. (4) and these results were in accordance with that reported by (23), they mentioned that this medium was BCYE agar with antimicrobial agents such as polymyxin B (that inhibits the growth of gram negative bacteria), vancomycin (inhibits the growth of gram positive bacteria) and anisomycin (which inhibits the growth of yeast and fungi), and differential dyes such as bromothymol blue and bromocresol purple. In addition to amino acid glycine which sufficiently inhibited the non- *Legionellaceae* bacteria and as a result greatly facilitated the isolation of *L. pneumophila* from specimens (9).





Fig . (1): Growth of *L. pneumophila* on BCYE Agar Medium .

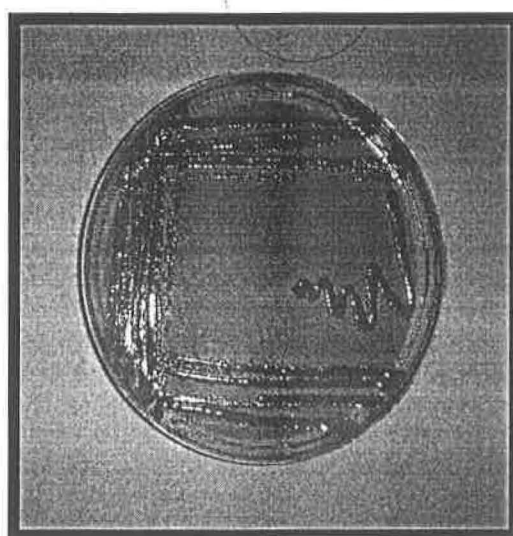


Fig. (2): Growth of *L. pneumophila* on Feeley Gorman Agar Medium.



Fig. (3): Growth of *L. pneumophila* on Yeast Extract Tyrosine Agar Medium .

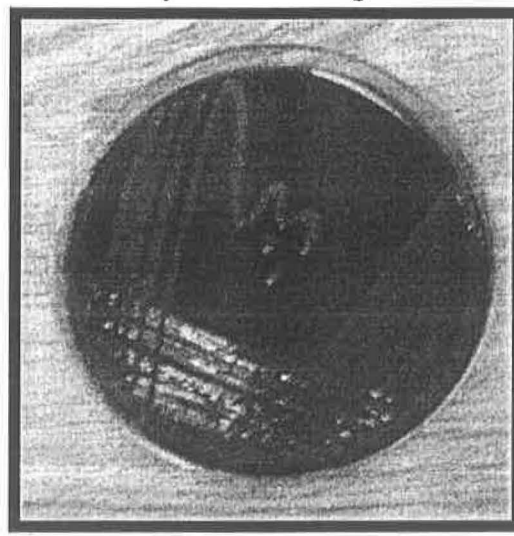


Fig. (4): Growth of *L. pneumophila* on MWY Agar Medium.

Each isolate was examined by Gram staining and the results of this study showed that this bacterium was non-spore forming, non capsulated, and poorly-stained gram negative rods (fig. 5). These results were agreed with that published by (21) .

*Legionella* is weakly stained with gram stains as it is revealed by (7), because of that Geimsa stain used in this study. In this case the bacteria were stained by blue-purple color (fig.6) and these results were in accordance with those reported by (5,10).

Also when smears of *Legionella* were stained with Acridine orange, it appeared as iridescence orange, small rods when examined

under fluorescent microscope (fig.7) and those results were in agreement with (17).

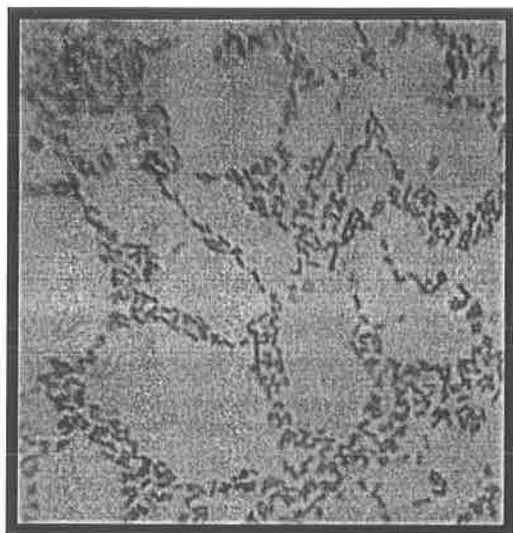


Fig. (5): *L. pneumophila* cells stained with Gram stain (1000X).

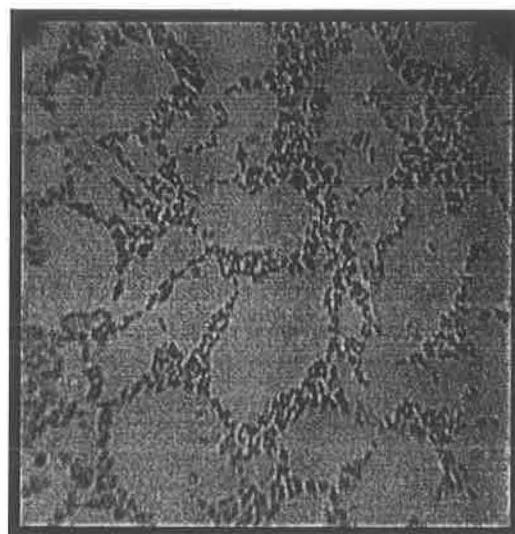


Fig. (6): *L. pneumophila* cells stained with Geimsa stain (1000X).

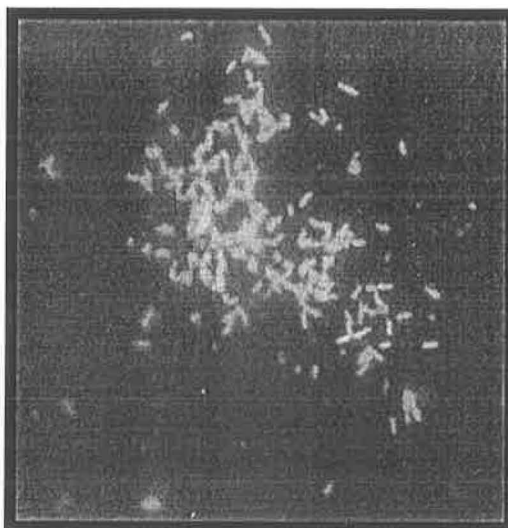


Fig. (7): *L. pneumophila* cells stained with Acridine Orange stain (1000X).

### Biochemical tests

Table 1 shows the biochemical characteristics of *L. pneumophila* isolated from clinical and environmental samples:

**Table 1: Results of biochemical tests for *L. pneumophila* bacteria under study.**

Biochemical tests	Results of clinical isolates	Results of environmental isolates
Oxidase test	+	+
Catalase test	+	+
Gelatinase test	+	+
Hippurate hydrolysis test	+	+
Starch hydrolysis	+	+
DNase test	+	+
Lipase and Lecithinase test	+	+
Protease test	+	+
Urease test	-	-
Nitrate reductase	-	-
Motility test	+	+
IMViC test	-	-

(+): Positive

(-): Negative

### Biotyping of *L. pneumophila*:-

From table 2 it was found that a total of 50 clinical and environmental isolates, 33 (66%) of isolates were located in biotype I depending on their ability to produce protease, DNase, and starch hydrolysis enzyme. While, 7 isolates in percentage (14%) were located in biotype II which produce protease and DNase enzyme. However, 5 isolates which represent (10%), were located in biotype III that gave positive response to protease and starch hydrolysis test. On the other hand, only one environmental isolate (2%) was located in biotype VI because this isolate has the ability to produce only protease enzyme. Whereas, 3 isolates with percentage (6%) were located in biotype V depending on their ability to produce DNase and starch hydrolysis enzyme. In addition, only one isolate of environmental isolates was located in biotype IV which was able to produce only DNase enzyme and gave a negative reactions to other two tests. In this study there is no isolate located in biotype IIV and IIIV.



Our results indicated that all isolates were distributed in six biotypes and most of them were located in biotype I because most of *L. pneumophila* isolates have the ability to produce these enzymes counted as virulence factor and have role in occurrence of infection.

**Table 2: Biotyping of *L. pneumophila* isolates.**

Test System	Biotypes							
	I	II	III	VI	V	IV	IIV	IIIV
Protease	+	+	+	+	-	-	-	-
DNase	+	+	-	-	+	+	-	-
Starch Hydrolysis	+	-	+	-	+	-	+	-
No. of clinical isolates(%)	15 75%	3 15%	1 5%		1 5%			
No. of environmental isolates(%)	18 60%	4 13.33 %	4 13.33 %	1 3.33%	2 6.67%	1 1.33%		
Total	33 66%	7 14%	5 10%	1 2%	3 6%	1 2%		

(+) : Positive

(-) : Negative

### Adherence assay:-

Our finding indicated that all isolates of *L. pneumophila* have the ability to adhere to the epithelial cells of the human buccal cavity as it appears in fig. (8) and these results were in agreement with that published by (24).

*L. pneumophila* is a facultatively intracellular pathogen that is able to adhere, infect, multiply within, and kill human macrophages as well as free-living amoeba (24). The ability of *L. pneumophila* to grow within mammalian cells, such as monocytes and alveolar macrophages, is essential for Pathogenicity in humans.

Furthermore, (25) demonstrated that interaction between intracellular pathogens and host cells are mediated through attachment of a bacterial ligand, such as a pilus, to a receptor on the surface of a host.

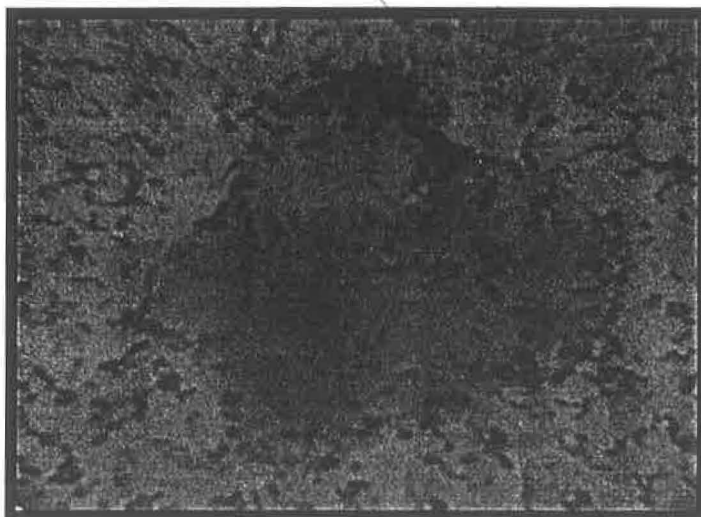


Fig. (8): Adherence of *L. pneumophila* to the epithelial cells of the human buccal cavity (1000X).

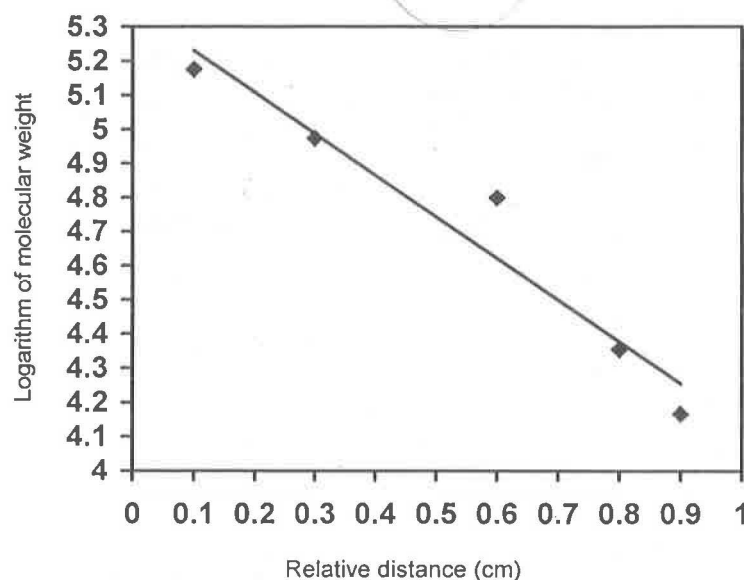
On the other hand, many researchers reported that *L. pneumophila* had a high ability to adhere to the host cells by specific receptors presented on the surface of the host cells and these receptors were proteins which differ from host to another (25,26).

#### Separation of LPS by using SDS-PAGE Electrophoresis:-

The LPS of clinical (lane B) and environmental (lane C) isolates of *L. pneumophila* were analyzed by SDS-polyacrylamide gel electrophoresis and the results of our study showed differences in the number and molecular weights of bands which were estimated depending on standard curve of standard proteins as it illustrated in table 3 and fig. (9).

**Table 3: The relationship between molecular weight and relative distance of standard proteins for determining the approximate molecular weight of LPS using SDS-PAGE technique.**

Standard Proteins	Molecular weight		Relative distance (cm)
	Kilo Dalton	Logarithm	
Γ -Globulin	150	5.1	0.1
Transferine	80	4.9	0.3
Pepsin	67	4.8	0.6
Trypsin	20	4.3	0.8
Lysozyme	14	4.1	0.9

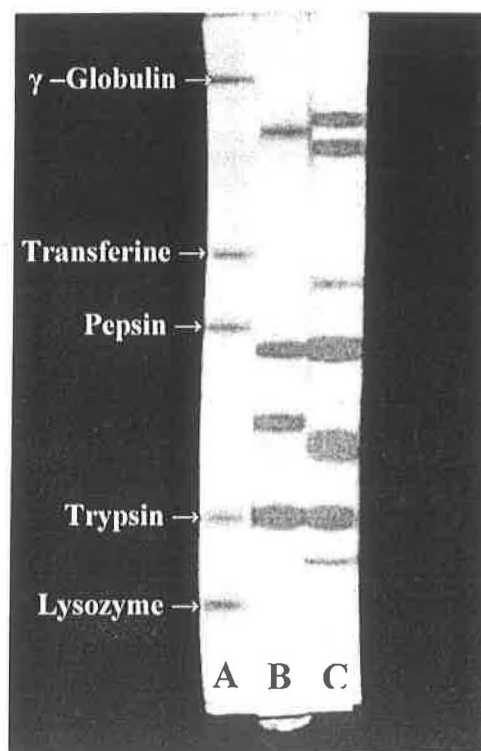


**Fig. (9): Standard curves for determination of the molecular weight using SDS-PAGE technique.**

From fig. (10) we found that the representative LPS profile of *L. pneumophila* (lane B) gave four bands with different molecular weight ranged (20 - 138) KD, whereas, the LPS of *L. pneumophila* (lane C) gave seven bands representing different molecular weights ranged from (27 - 144) KD and this results were similar to that obtained by (27) when they revealed that the migration characteristics of LPS from *L. pneumophila* strains were very distinctive .

Moreover,(27) demonstrated that the individual differences in the LPS banding patterns after SDS -PAGE were due to the different numbers of representing units in the O-site chain of the LPS molecule and it has been suggested that exhibited different LPS profiles in SDS -PAGE are likely to possess serologically distinct O -antigens .

Furthermore, LPS of *L. pneumophila* is considered a factor mediating pathogenicity. It is the major immunodominant antigen and represents the basis for the classification of serogroups (28,29). In contrast to enterobacterial LPS activation it has been shown that *Legionella* LPS is able to activate both classical and alternative complement pathway (28). Due to the exceptional chemical structure of the *L. pneumophila* LPS, it is likely that the molecule participates in a number of essential *Legionellae* capabilities, such as adaptation to various environmental challenges (29).



**Fig. (10): Separated protein and LPS bands by using SDS-PAGE technique :**

- Lane (A): Standard proteins bands.
- Lane (B): Clinical isolate bands.
- Lane (C): Environmental isolate bands.

However, (30) revealed that the *Legionella* strains showed large number of broader bands, representing different molecular weights ranged from (14-95)KD and also they typed (430) strains of *L. pneumophila* to different serogroupe depending on LPS profile SDS - PAGE and silver staining. by using.

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