RESULTS

The study was carried out from June 2001 to April 2002 on 150 environmental samples and 100 clinical samples from inpatients suffering from clinically diagnosed nosocomial pneumonia at Benha University Hospital and classified as follows.

1- Environmental Samples:

- 100 Samples from faucets of water.
- 20 samples from air conditions.
- 20 samples from showers.
- 5 samples from respirators.
- 5 samples from humidifiers.

2- Clinical Samples:

The clinical samples included sputum, transtracheal aspirate and pleural effusion. The samples were collected from 100 inpatients with different age groups,and suffering from different illnesses complicated by pneumonia, as shown in tables 3&4&5 (fig4&5) from Benha University Hospital departments (I.C.U., Chest department, General Medicine department, Surgery department, Pediatric department, Gastroentrology department and Cardiology department).

i) Results of Bacteriological Examination of Collected Samples for *L. pneumophila:*

Ten strains (4%) of *L.Pneumophila* were isolated from 10 samples out of all collected environmental and clinical samples (250 samples) (fig.6). All the collected samples were bacteriologically examined by

culturing on buffered charcol yeast extract agar (BCYE), followed by biochemical identification .

Results of Bacteriological Examination of Environmental Samples

Eight strains (5.3%) of *L. Pneumophila* were isolated from 8 environmental samples out of 150 (fig.7) (strain No 1-8). The positive environmental samples were distributed as follows: 5 (5%) from 100 faucets, 2 (10%) from 20 air conditions, and 1 (5%) from 20 showers (table 6&7&8)(fig 8&9).

The relation between the number of the positive samples and the time of their collection was shown in table (9). The number of the positive samples was more during August followed by July and finally by September.

It was found that 4 of the 8 positive environmental isolates (50%) were isolated by swab method, 2 (25%) were isolated by water sampling method and 2 (25%) were isolated by membrane filtration method (table 10)(fig.10).

Results of Bacteriological Examination of Clinical Samples

Two strains (2%) of *L.pneumophila* were isolated from two clinical samples out of 100 (fig.11) (strain no. 9,10) .One strain (1%) was isolated from a male patient 67 years old who had hepatocellular carcinoma and developed pneumonia 10 days after his admission to the hospital for investigation. The other positive sample (1%) was obtained from a male patient 37 years old with chronic leukemia and developed pneumonia 6 days after his admission to the hospital for blood transfusion and treatment the complication of the bleeding attack (table 11).

One of the two clinical *L.pneumophila* isolates was from one patient out of 28 (3.6%) with no history of chemotherapy, the other strain from another one patient out of 7 (14.3%) with history of repeated courses of chemotherapy as shown in tables 12&13 .

b) Identification of *Legionella pneumophila:*

The organism was presumptively identified as legionella by its growth requirements, morphology and biochemical characteristics and definitively placed in the genus legionella by serotyping. The cultural characters of the organism was as follows: Legionella like colonies were observed on BCYE agar containing cysteine with an antibiotic supplement consisting of polymyxin B, anisomycin and vancomycin at 37°C.

- Growth was enhanced slightly on BCYE by incubation with added 2.5% CO_2 and humidity.
- *L. Pneumophila* colonis on BCYE agar were greyish white in colour, 1-4mm in diameter, rounded, convex with an entire edges and some strains were difficult to pick (table 14 & fig 12).
- Microscopic examination showed pleomorphic Gram negative rods faintly stained, approximately 0.5-2 μm in size with characteristic tapered shape. (fig. 13).
- Biochemical reactions showed positive catalase test in 8 out of 10, positive oxidase, positive starch hydrolysis tests and positive gelatin liquefaction in all the isolated strains (10) as shown in table 15.
- Definitive identification of *Legionella Pneumophila* isolates was done by latex agglutination test. Latex agglutination test allowed grouping of *Legionella Pneumophila* into 2 serogroups ; S_1 and S_2. In this study 6 of positive *L. Pneumophila* isolates were related to S_1 (strains no. 2,

5,6,7,9,10) and the remaining 4 positive *L. Pneumophila* isolates were related to S_2 (strains no. 1,3,4,8) as shown in table 16.

c) Results of Genotyping of Isolated Strains of *L.pneumophila* by Restriction Endonuclease Analysis(REA).

The DNA of the isolated strains (10) were digested and prepared for REA. These strains were derived from 8 environmental samples (fig 14, lanes 1-5 from faucets& lanes 6,7 from airconditions & lane 8 from shower),and 2 isolates from 2 patient who devoloped NP after thier admission to Benha Univesity Hospital (fig14,lanes 9&10).

Four patterns of genotyping had been recognized and designated as A&B&C and D patterns.

The endonuclease electrophoretic digest patterns of the two patients isolates and the faucet strains from General Medicine departement at Benha University (fig.14& lanes3&5&9&10) are indistinguished from one another in EcoRI and HindIII digests pattern D (fig.14). As identical or nearly identical restriction endonuclease profiles were observed in these strains of different *L.pneumophila* serogroups. Within serogroup1 (S1), three very different restricted digested patterns were observed among the studied 6 strains of serotype 1.

The other environmental isolates have unique electrophoretic digestion pattern. Pattern A (fig 14& lanes 1&7), pattern B (fig 14 & lanes 2&6) and pattern C (fig.14 & lanes 4&8).As the strains were distinguishable from one another in EcoRI and Hind III digests.

d) Results of Studying The Cytopathic Effect of *L. Pneumophila* Isolates in Vero Cells:

The effect of the 10 *L. Pneumophila* isolates in vero cells were assayed.The affected cells appeared rounded, clumps of granular cells surrounding circumscribed zone of cell lysis. The isolated 10 strains of *L. Pneumophila* induced the above effect in monolayer tissue culture within 24 hours, but the cytopathic effect increased with time (after48 hours).

The study showed that after one day of inoculation, 2 strains of *L. Pneumophila* isolates produce cytopathic effect in about 75% of the cells of monlayer tissue culture, 6 strains induce cytopathic effect in about 50% of the cell of monolayer tissue culture and 2 strains induce cytopathic effect in about 25% of the cells (table 17).

The study also showed that after two days of inoculation 7 strains of *L. Pneumophila* isolates induced cytopathic effect in more than 90% of the cells of monolayer cell culture and 3 strains induced cytopathic effect in about 75% of the cells of monolayer cell culture (table 18) & (fig.15&16&17).

Table (3): Sex distribution among studied clinical groups .

Sex / Age (years)	Male		Female		Total	
	No	%	No	%	No	%
(0 - 10)	8	11.76	5	15.62	13	13
(11 - 20)	6	8.82	3	4.41	9	9
(21 - 40)	9	13.23	4	12.50	13	13
(41 - 60)	21	30.80	8	25	29	29
above 60	24	35.29	12	37.5	36	36
Total	68	100	32	100	100	100

There is high significant difference in the sex among the studied groups regading to NP.

Table (4): Mean and range of age among studied clinical groups

Age (years)	No	$\overline{X}$	S.D.	Range	
				Minium	Maxium
Group I (0-10)	13	0.369	$\pm$ 0.652	3 (days)	10
Group II (11-20)	9	15.1	$\pm$ 2.934	11	20
Group III (21-40)	13	31.15	$\pm$ 6.335	22	40
Group VI (41-60)	29	51	$\pm$ 5.849	41	60
Group V above 60	36	66.468	$\pm$ 13.425	62	73

$\overline{X}$: Arthrmatic mean

S.D.: standard deviation

Table (5): High and low risk groups of illness as regard to *L. Pneumophila* from clinically diagnosed NP.

High risk group			Low risk group			Z	P
Illness	**No**	**%**	**Illness**	**No**	**%**		
Tumor	35	38.04	Surgery	3	37.5		
Renal faliure	23	25	Chronic obstructive pulmonary disease (COPD)	1	12.5		
Mechanically ventilated	15	16.30					
Coma	9	9.76	Rheumatic heart disease	1	12.5		
Premature	10	0.86	Malnurished infants	3	7.5		
Total	92	100		8	100	8.4	<0.001 (HS)

H.S.: Highly significant

High risk group is highly significant than low risk group.

Figure 4 :Sex distribution among studied groups.

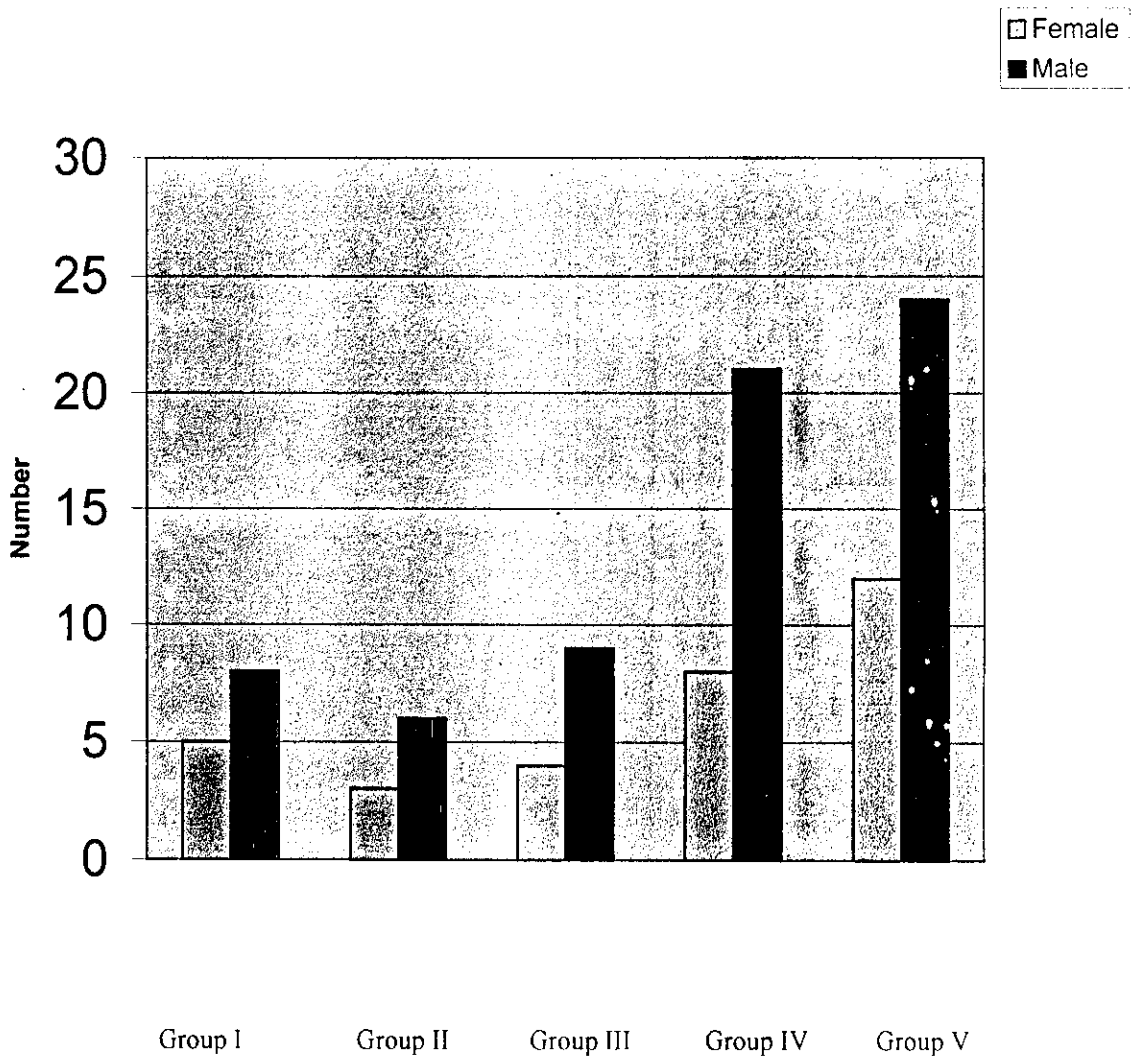

Figure 5 :Mean age among studied groups.

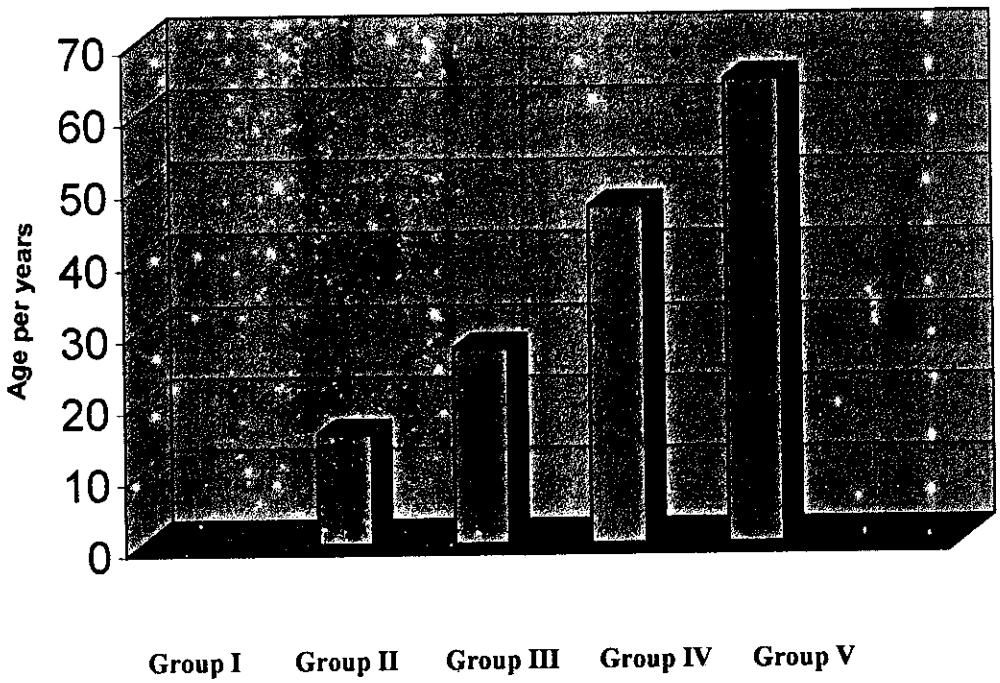

Figure 6 : Positive *L.pneumophila* isolates in total tested samples (evironmental and clinical sampls).

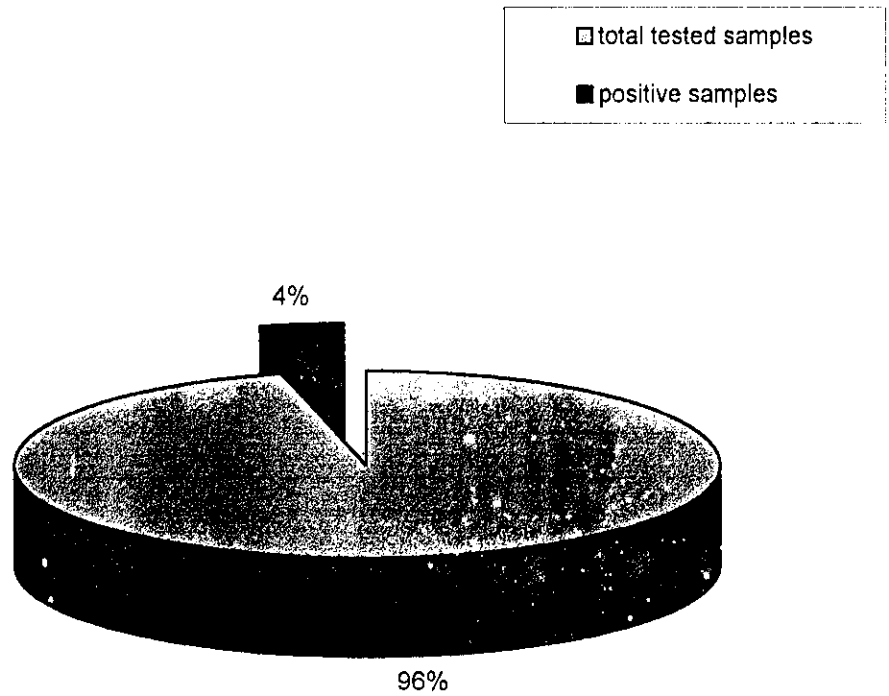

Figure 7 : Positive *L.pneumophila* isolates in tested environmental samples.

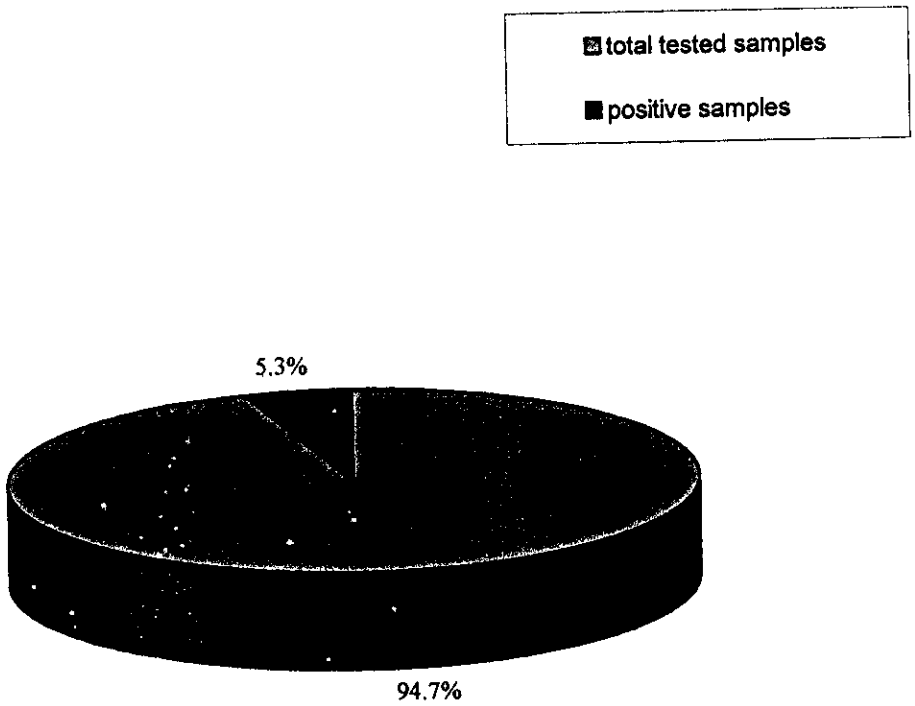

Table (6):Distribution of *L. Pneumophila* in the collected environmental sample.

Source	Positive samples		Negative sample		Total No. of tested samples	
	No	%	No	%	No	%
Faucets	5	5	95	95	100	100
Air conditions	2	10	18	90	20	100
Showers	1	5	19	95	20	100
Respirators	0	0	5	100	5	100
Humidifiers	0	0	5	100	5	100
Z_1	0.214		P_1		> 0.05 (N.S)	
Z_2	0.164		P_2		> 0.05 (N.S)	

N.S.: Non significant

There is no significant differences in distribution of *L. pneumophila* between air conditions, faucets and showers.

Table (7): Pattern of distribution of *L. pneumophila* in the positive environmental samples.

Source	No. Positive sample	%	Z	P
Faucet	5	62.5	-	-
Air conditions	2	25	1	> 0.05 (Ns)
Showers	1	12.5	1.265	> 0.05 (Ns)

NS: Not significant

Faucets samples found to be the main source for isolation of *L.pneumophila* (62.5), followed by air condition samples (25%), then showers samples (12.5%).

Table (8): *Legionella pneumophila* isolates from environmental samples.

Department	Sources				
	Faucet	Air condition	Showers	Respiratory	Humidifiers
Chest department	1	-	-	-	-
Internal medicine Department	2	1	1	-	-
Surgery department	1	-	-	-	-
I.C.U.	-	1	-	-	-
Pediatric department	-	-	-	-	-
Gastroentrology department	1	-	-	-	-
Cardiology department	-	-	-	-	-
Total	5	2	1	-	-

4 positive samples were collected from Internal Medicine department, one positive sample from Chest department, one positive sample from Surgery department, one positive sample from I.C.U. and one positive sample from Gastro Entrology department.

Figure 8 : Distribution of *L.pneumophila* in the collected environmental samples.

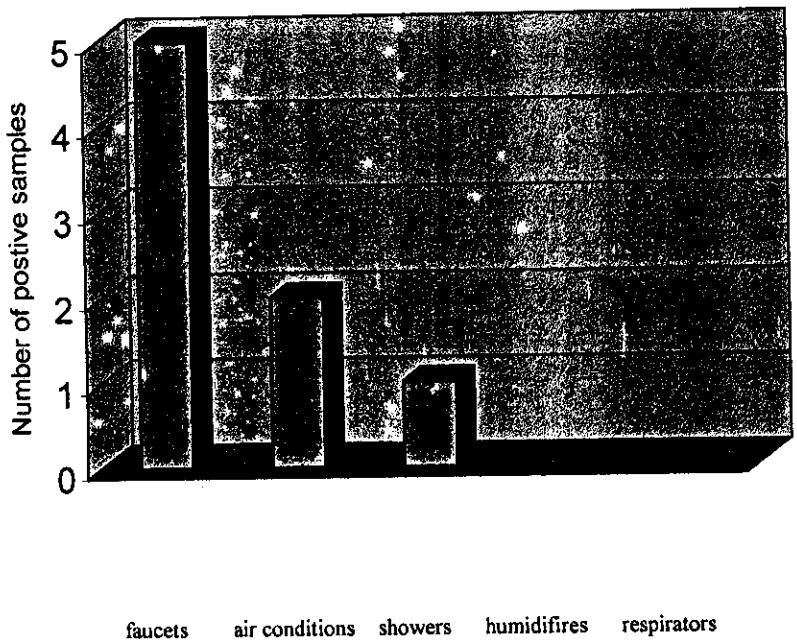

Figure 9 : Pattern of distribution of *L.pneumophila* in positive environmental samples.

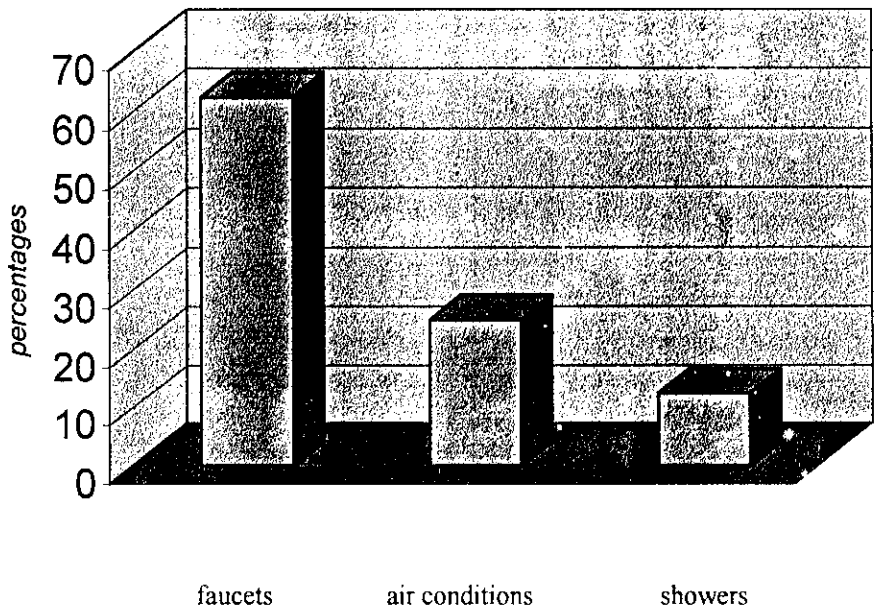

Table (9): Incidence of isolation of *L.pneumophila* from different environmental sources in different months.

Mouth	No of sample	*L.pneumophila* isolates		Significance against August	
		No	%	Z	P
June	15	0	0	-	-
July	43	3	7	0.474	> 0.05 (N.S.)
August	50	4	8	-	-
September	25	1	4	0.183	>0.05(N.S)
October	11	0	0	-	-
November	6	0	0	-	-

N.S.: Non-significant difference between July, August and September.

Table (10): Percentage of positive *L. pneumophila* isolates from environmental sources according to sampling methods.

Sampling	No. of positive samples	%
Swabing method	4	50
Water sampling method (after concentration)	2	25
Membrane filtration method	2	25

Swab method yeilded the highest percentage of *L. pneumophila* isolates (50%), followed by water sampling method (25%) and filtration methods (25%).

Figure 10: Percentages of *L. pneumophila* isolates from positive environmental sources according to sampling methods.

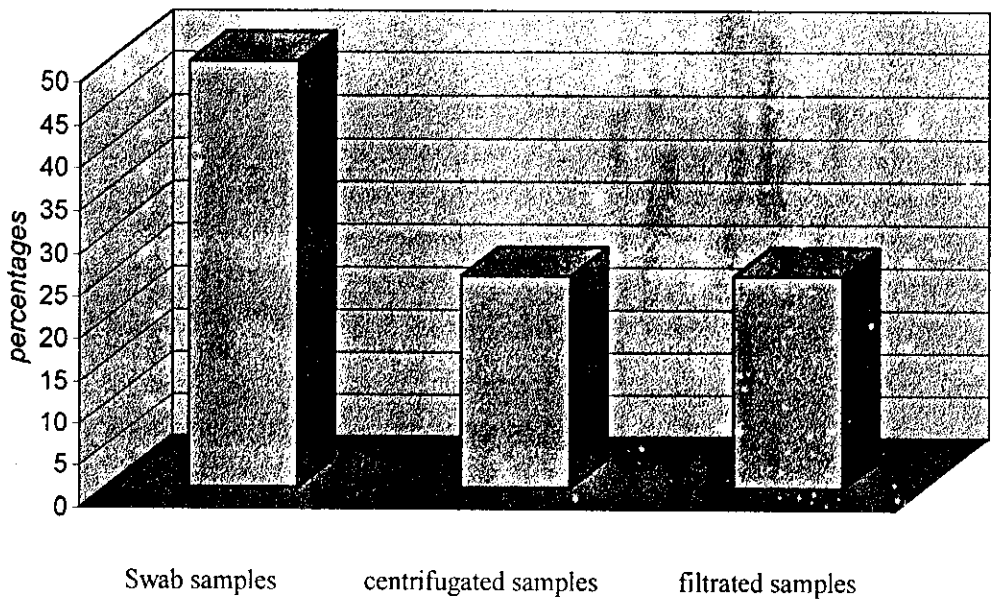

Table (11): Distribution of *L. pneumophila* in the studied clinical samples.

Total No. of samples	Positive samples for *L. pneumophila*		Negative samples for *L. pneumophila*		Z	P
	No	%	No	%		
100	2	2	98	98	9.6	<0.001 (H.S.)

H.S: Highly significant.

Table (12): Incidence of isolation of *L. pneumophila* in patients with malignant illnesses regards to chemotherapeutic treatment.

Patients with chemotherapeutic treatment			Patients with no chemotherapeutic treatment			Z	P
No.of tested patients	Positive for *L.pneumophila*	%	No.of tested patients	Positive for *L.pneumophila*	%		
7	1	14.3	28	1	3.6	0.831	> 0.05 (Ns)

NS: Non significant.

There was no significant difference between patients with malignant illnesses receiving chemotherapeutic treatment and those with no chemotherapeutic treatment.

Figure 11 :Positive *L.pneumphila* isolates in tested clinical samples.

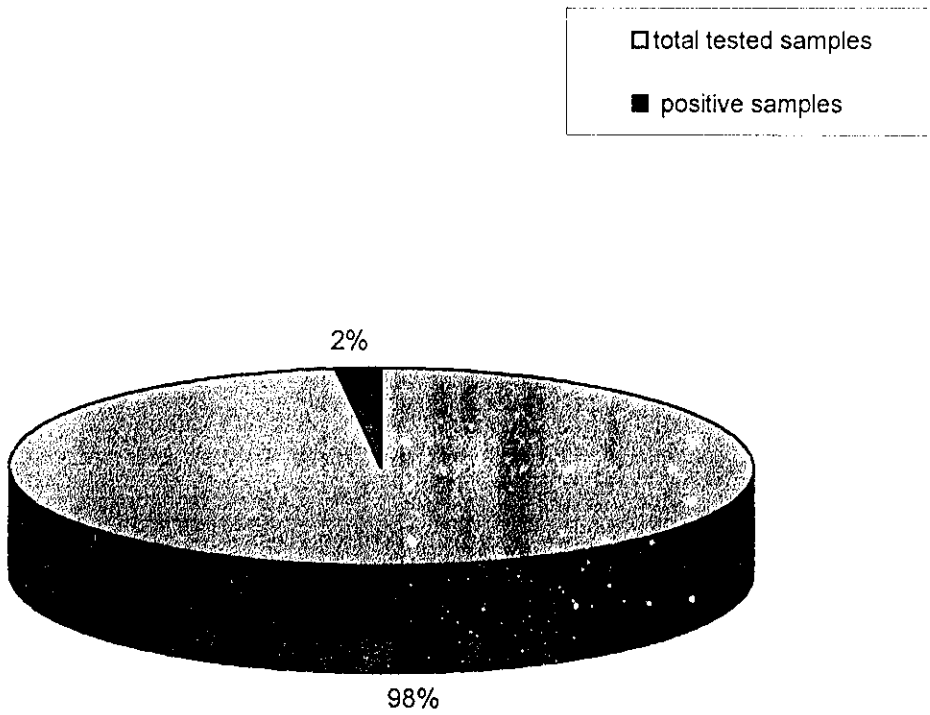

Table (13): Tumors versus other high risk factors regarding to *L. pneumophila* isolation.

Illnesses	No.of tested patients	No.of positive cases for *L. pneumophila*	%	Z	P
Tumors	35	2	5.8	1.48	> o.o5
Renal faliure	23	-	0	1.48	> o.o5
Mechanically ventillated	15	-	0	1.48	> o.o5
Coma	9	-	0	1.48	> o.o5
Pre mature	10	-	0	1.48	> o.o5

There was no significant difference between Patients with malignant illnesses and patients with other risk factors for isolation of *L. pneumophila.*

Table (14): Morphological character of *legionella pneumophila* isolates.

Sample No.	Colonial morphology of positive samples
1,6,7	Greyish white 2-4 mm in diameter, rounded, convex and difficult to pick off.
2,3,5,9,10	Greyish white 1-3mm in diameter, rounded, convex with an entire edge and stringy.
4,8	Greyish white 1-2 mm in diameter, rounded, convex with entire edge.

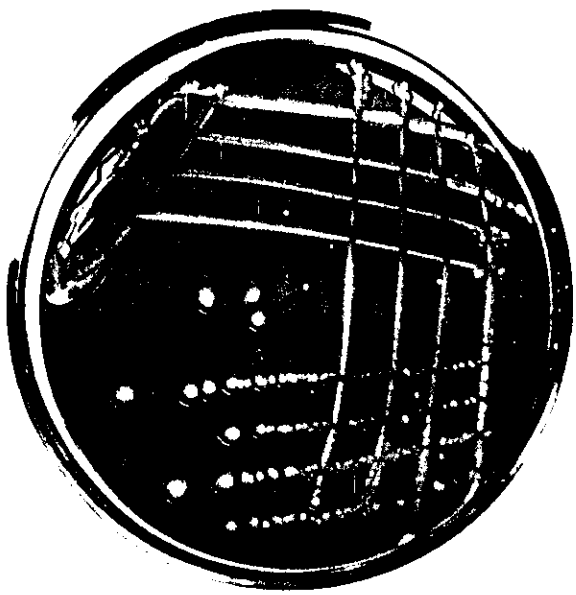

Figure 12:*L.pneumophila* colonies on BCYE agar.

Figure 13:Gram stain of *L.pneumophila* (in culture).

Table (15): Biochemical reactions of positive *Legionella pneumophila* isolates.

Sample	Oxidase	Catalse	Gelatin liquefaction	Starch hydrolysis
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	-	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	+
9	+	-	+	+
10	+	+	+	+

All isolated strains were positive oxidase, positive gelatin liquefaction, positive starch hydrolysis. Two strains were catalse negative.

Table (16): Serological identification of *Legionella pneumphila* by latex agglutination test.

Sample No.	Sero group
1	S_2
2	S_1
3	S_2
4	S_2
5	S_1
6	S_1
7	S_1
8	S_2
9	S_1
10	S_1

S_1: *L. Pneumophila* (sero group 1)

S_2: *L. Pneumophila* (sero group 2-14)

6 strains of *L. pneumophila* are S_1 and 4 strains are S_2

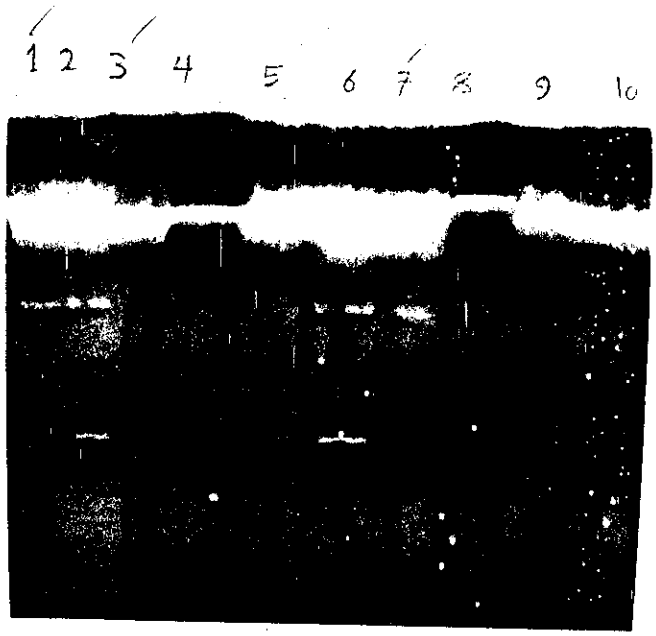

Figure 14: Genotyping of *L.pneumophila* isolates by REA

Lanes (1,2,3,4,5) : The digestion pattern of isolated strains from faucets samples.
Lanes (6,7) : The digestion pattern of isolated strains from air conditions samples.
Lanes (8) : The digestion pattern of isolated strain from showers samples.
Lanes (9,10) : The digestion pattern of isolated strains from clinical samples.
****Lanes (3,5,9,10) had similar electrophoretic pattern, Lanes (1,7) had similar electrophoretic pattern,Lanes (2,6) had similar electrophoretic pattern, Lanes (4,8) had similar electrophoretic pattern.***

Table (17): Cytopathic effect of *L. Pneumophila* strains in vero monolayer after one day of inoculation.

Sample No.	Percentage of cells affected
1	≤ 50%
2	≤ 50%
3	≤ 75%
4	≤ 25%
5	≤ 50%
6	≤ 75%
7	≤ 25%
8	≤ 50%
9	≤ 50%
10	≤ 75%

After one day of inculation, 2 strains of *L. Pneumophila* isolates produce cytopathic effect in 75% of the cells of monlayer tissue culture, 6 strains induce cytopathic effect in 50% of the cell of monolayer tissue culture and 2 strains induce cytopathic effect in 25% of the cells.

Table (18): Cytopathic effect of *L. Pneumophila* strains in vero monolayer after two day of inoculation.

Sample No.	Percentage of cells affected
1	≤ 75%
2	≥ 90%
3	≥ 90%
4	≤ 75%
5	≥ 90%
6	≥ 90%
7	≥ 90%
8	≤ 75%
9	≥ 90%
10	≥ 90%

After two days of inculation 7 strains of *L. Pneumophila* isolates induced cytopathic effect in more than 90% of the cells of monolayer cell culture and 3 strains induced cytopathic effect in 75% of the cells of monolayer cell culture.

Figure 15 :Normal Vero cell line before inoculation.

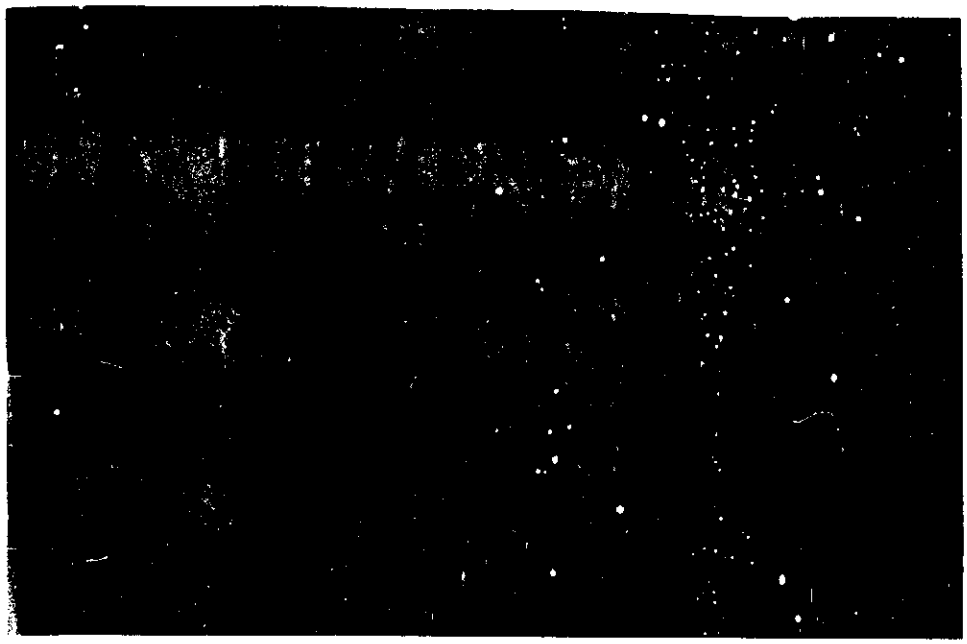

Figure 16 : Cytopathic effect of *L.pneumophila* in Vero cell line.
(cells rounding with zone of cell lysis)

Figure 17: Cytopathic effect of *L.pneumophila* in Vero cells.

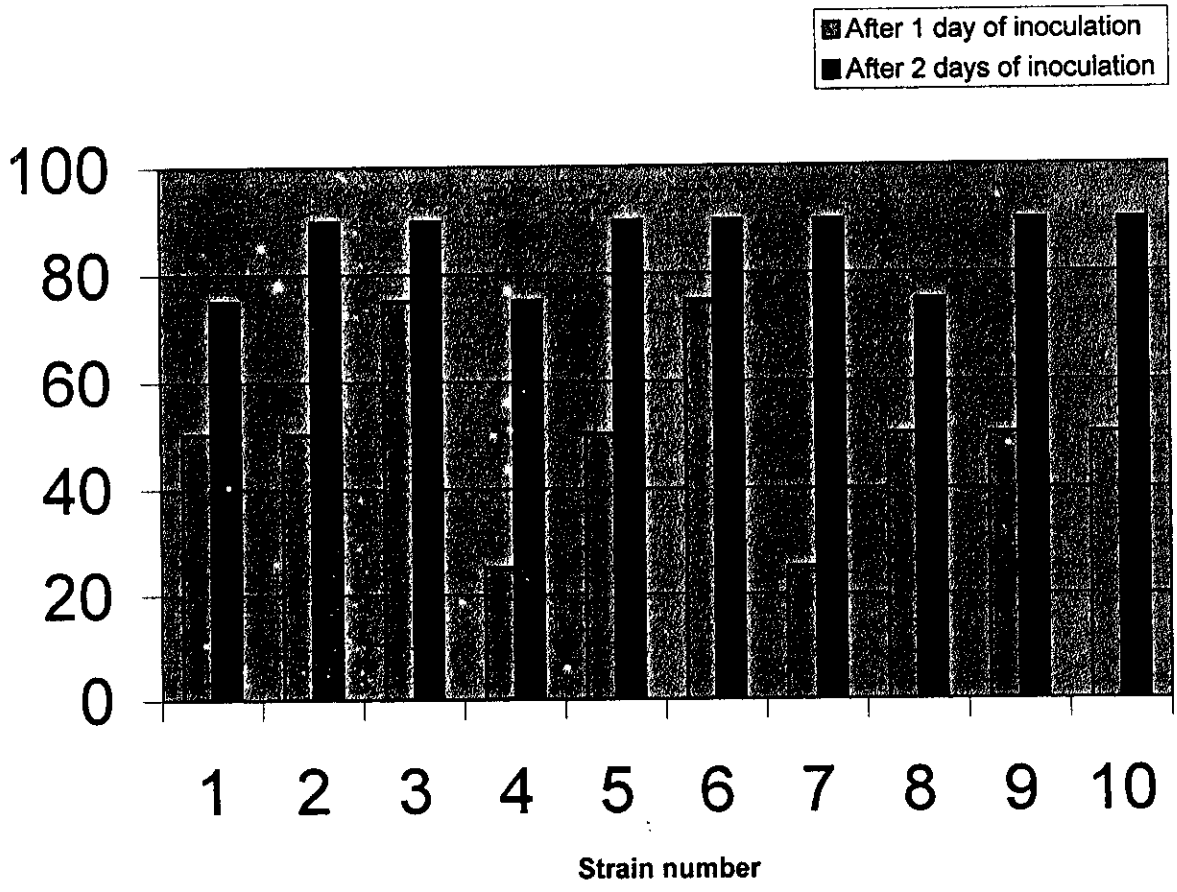

Table (19) : Grades of virulence of *L. pneumophila* isolates in vero cells versus to genotype pattern.

Grade of virulence	Patterns of genotype			
	A Strain No.	B Strain No.	C Strain No.	D Strain No.
High	-	6	-	3 & 10
Modrate	1	2	8	5 & 9
Low	7	-	4	-

- High virulence : affect ≥75% of the cells.
- Modrate virulence : affect ≤ 50% of the cells.
- Low virulence : affect ≤ 25% of the cells.