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STUDIES ON DETECTION, DIAGNOSIS AND CONTROL OF *LEGIONELLA PNEUMOPHILA* IN DIFFERENT WATER SAMPLES

[38]

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ABSTRACT

A number of 192 samples (180 water & swab specimens and 12 patient sputa) were chosen from different sites of Mansoura University Hospitals for detection and diagnosis of *Legionella pneumophila*. Out of the 192 samples, 148 were positive by culture method and non-specific staining technique such as gram staining and biochemical analysis. In our trials for diagnosis of *Legionella pneumophila*, we found that 107 of 148 samples were positive by slide agglutination test and 110 samples were positive by direct immunoflourescence assay, while 130 samples were positive by polymerase chain reaction (PCR) technique which proved to be the most specific and sensitive technique for diagnostic investigation of *L. pneumophila*. The results show that the four disinfectant procedures (chlorine, ozone, U.V. light and heat) were effective in eradicating *Legionella pneumophila* from different water samples. Both UV light and heat (60ºC) produced a 5 log kill in less than 1 h. In contrast, both chlorine and ozone required 5 h of exposure to produce a 5 log decrease. Neither turbidity nor the higher temperature of 43ºC impaired the efficacy of any of the disinfectant methods.

Keywords: Sputa, *Legionella pneumophila,* U.V. light, Disinfectants, Immunoflourescence assay.

INTRODUCTION

The genus *Legionella*, Family Legionellaceae contains at least 22 species most of which have yet to be isolated from clinical specimens **(Campbell *et al* 1984; Gondaira & Sugiyama, 1996 and Buising *et al* 2001).** Members of this genus are nutritionally fastidious, gram-negative, non-sporeforming bacilli and most not all species are motile by virtue of one or two monopolar flagellae **(Hart and Makin, 1991).**

The legionellae are the causative agents of legionnaires disease, a multisystem disease manifested primarily as pneumonia **(Meyers, 1983; Hart & Makin, 1991; Formica *et al* 2000 and Gracia *et al* 2003).**

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The major reservoirs of *Legionella* species appear to be fresh-water sites, air-conditioning units and various potable water systems **(Fliermans, 1983 and Torrijos *et al* 1995).**

The only criterion used to distinguish species of *Legionella* has been DNA hybridization studies **(Jones and Hebert, 1979)** and phenotypic traits such as pigmentation, autofluorescence, gelatinase production, hippurate hydrolysis and cellular fatty acids. Serotyping plays a major role in species and subgroups identification **(Bibb *et al* 1981 and Formica *et al* 2001).**

There are many methods of varying efficacy for controlling legionellae in hospital hot water systems. They fall into three categories : physical, chemical, and good plumbing practice such as heat, UV, sonication, draining and flushing with compressed air, sodium hypochlorite and ozone **(Muraca *et al* 1995 and Hoebe and Kool, 2000).**

The present study was conducted to find out reliable methods for detection, diagnosis and control of *Legionella pneumophila* in different water system in Mansoura University Hospitals.

MATERIAL AND METHODS

Sampling

One hundred and twenty tap water samples from bathrooms, twenty water samples, forty swab samples and twelve sputum samples were collected under complete aseptic conditions from different sites of Mansoura University Hospitals **(Table 1).** Samples were transported immediately in sealed containers to the laboratory. Two liters of water were collected upon two occasions, one liter each, during the period from October, 1996 to April, 1997.

The positive control reference of all laboratory diagnostic techniques for *L. pneumophila* serotype 1 is strain Philadelphia 1 (ATCC 33152) that has been kindly supplied by Prof. Dr. Helmy T. El-Zanfaly, Water Pollution Department, National Research Center, Dokki, Egypt.

Cultivation of Legionellas

The recommended culture medium is BCYE agar medium (buffered charcoal-yeast extract agar); this is proved more satisfactory than the enriched blood agar media described by **Greaves (1980).**

Methodology

The following laboratory investigations were done to all specimens and to positive control references:

1- Methods of Isolation and Culture Conditions

Isolation of *L. pneumophila* from water and water systems was carried out using the methods of **Arnow *et al* (1985) and Ribeiro *et al* (1987).**

2- Morphological characteristics

Including colonial characteristics, shape & size of cells, and staining reaction. The isolation of a single bacterium and formation of separate colonies was carried out according to the technique of **(Boyd and Marr, 1980).**

3- Biochemical reactions

3-1. Hipurate hydrolysis, was tested following the method adopted by **Baer and Dasis (1981).**

3-2. Catalase test, was carried out following the procedures described by **Boyd and Marr (1980).**

3-3. Gelatin liquefaction test, was performed as described by **Boyd and Marr (1980).**

3-4. Starch hydrolysis test, was carried out as described by **Baker and Breach (1980).**

3-5. Oxidase test, was carried out as described by **Smith (1980).**

4- Antibiotic sensitivity test

This can be demonstrated by disk diffusion method **(Finegold and Martin, 1982).**

5- Serotyping of Legionella by Latex Agglutination Test (Mast Diagnostica)

Agglutination test was performed by the method described by**(Harrison *a*nd Taylor 1988**).

6- Direct immunofluorescence test (DFT)

The direct immunofluorescence test (DFT) was carried out using direct immunofluorescence for the detection of *Legionella pneumophila* with monoclonal antibodies (Freka fluor *L. pneumophila* MAB DFT).

7- Polymerase Chain Reaction (PCR) Technique

Diagnosis of *L. pneumophila* by PCR was carried out according to the method of **Koide and Saito (1995).** The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mg of gelatin per liter, deoxy nucleotide triphosphatase (each at a concentration of 200 μM), primers (each at a concentration of 50 pM), and 2.5 μ of Taq DNA polymerase.

8- Efficacy of disinfectants on *L. pneumophila* from Potable Water System

To compare the efficacy of ozonation, UV light, hyperchlorination and heat disinfection, a model plumbing system was constructed of copper piping, brass spigots, Plexiglass reservoir, electric hot water tank, and a pump **(Muraca *et al* 1995).** *Legionella pneumophila* was added to the system at 10^7 CFU/ml. Each disinfectant was tested under three conditions:

i) Nonturbid water at 25ºC.
ii) Turbid water at 25ºC.
iii) Nonturbid water at 43ºC.

Before each experimental run, the system was filled with hot sterile tap water 80ºC, each sample port was purged and the water was recirculated for no longer than 24 h. This served to flush the system of all bacterial contaminants and provided a sterile baseline environment.

RESULTS AND DISCUSSION

Detection and Isolation of *Legionella pneumophila* in Water Systems and Patient Sputa

In the present investigation a number of 180 water samples & 12 sputum samples were collected from different sites of Mansoura University Hospitals and cultured on BCYE agar medium, according to the methods described by **Ribiero *et al* (1987).** The results **(Table 1)** show that all samples from air conditioning sites, patient sputa (from patients with liver disease and patients subjected to renal transplantation) as well as water specimens from air conditioners showed 100% growth of *L. pneumophila* with mean viable count of 3.2 X 10^2, 4 X 10, 1.4 X 10 and 1.8 X 10^3 CFU/100 ml, respectively. These results indicate that, *L. pneumophila* is more frequent in sputa & water samples from air conditioning sites rather than water samples from mixer taps and water samples from water taps. **Jaulhac *et al* (1998)** reported that Legionnaires disease occurred in at least 25% of immunosuppressed patients exposed to aerosolized tap water containing Legionella with viable count of *L. pneumophila* 100 CFU/liter, whereas no cases were detected among 160 non-immunosuppressed patients who were similarly exposed.

Table 1. The percentage incidence of *Legionella pneumophila* in different samples.

No. of Sites	% of Positive growth	No. of CFU/100ml
20 Hot water from mixer taps	95%	2.7×10^4
20 Cold water from mixer taps	75%	1.2×10^3
20 Cold water from showers	60%	6.4×10^2
20 Hot water from showers	80%	3.0×10^3
20 Hot water from water taps	85%	8.0×10^3
20 Cold water from water taps	45%	1.0×10^2
20 Water specimens from air condition	100%	1.8×10^3
10 Swabs from sink and bath hot tap	95%	6.4×10^3
10 Swabs from sink and bath cold tap	90%	5.0×10^2
10 Swabs from shower roses	90%	4.2×10^3
10 Swabs from air condition sets	100%	3.2×10^2
8 Liver disease patients sputa	100%	4.0×10
4 Renal transplantation	100%	1.4×10

Morphological features of the Isolated Bacterium

The reference strains of *L. pneumophila* produces, on buffered charcoal-yeast agar medium (BCYE), circular colonies having smooth glistening surface, entire crenated edges, soft butyrous consistency with grey to grey-blue colorful appearance. The bacterial isolates that exhibit more or less similar characteristics are considered as presumptive positive *L. pneumophila* **(Fig. 1 a and 1b).**

The gram stain reaction shows negative, non-sporeforming short rods, they range in size and shape from uniform small bacilli 0.5 by 2.0 µm diameter by 100 µm length **(Fig. 2).**

Biochemical characteristics

The results revealed that, the isolates obtained from water samples, swab and patient sputa, showed positive hipurate hydrolysis, positive to catalase test, gelatin liquefact, starch hydrolysis and oxidase test. These tests indicate that these isolates belong to *L. pneumophila*.

Antibiotic sensitivity test

Antibiotic sensitivity test **(Fig. 3 a & b)** revealed that *L. pneumophila* was highly sensitive to chloramphenicol (C), erythromycin (E) and gentamycin (CN) followed by doxycycline hydrochloride (Do), rifampicine (RA) and cephalexin (CL). This is in agreement with **Smith *et al* (1997),** who found that the MIC for the *L. pneumophila* isolate of chloramphenicol, erythromycin and rifampicine was 0.3, 0.5 and 0.001 mg/L, respectively. In another study, **Higa *et al* (1998)** stated that, the MIC for the *L. pneumophila* isolate of gentamycin, doxycycline hydrochloride and cephalexin was 0.02, 0.003, 0.001 mg/L, respectively.

Serotyping

Serotyping was performed using agglutination test and direct immunofluorescent techniques. Only 107 of 148 positive sample by culture were positive by agglutination test **(Tables 2 & 3).** We found that 127 samples were positive by this test for serotypes (1-14), the maximum no. were positive for serotype 9 (14/127≅ 11%) and the minimum no. were positive for serotype 13 (1/127 ≅ 0.78%). In this study, serotype 6 was not appear by using this technique because agglutination tests measures primarily IgM antibody. Therefore, they may lack sufficient sensitivity for immunodiagnostic assay **(Miyamato *et al* 1995). Wilkinson and Fikes (1990)** reported that agglutination test is simple and specific and requires no expensive equipment or conjugates.

The direct immunofluorescence study showed green fluorescence bacilli **(Fig. 4),** 110 out of 192 samples were positive to this test, with high positive rate in case of serotype 1 (17.27%), and less positive rate in serotype 14 (1.8%) **(Table 4).**

Polymerase chain reaction (PCR)

In our trials to examine the best and rapid method for *L. pneumophila* diagnosis, we examined the application of the polymerase chain reaction (PCR) for the diagnosis of *Legionella*, we found that 130 of 148 positive samples by culture were positive by PCR and this ratio is higher than the DFT (110). We use two specific primers synthesized according to the reported *L. pneumophila* mip gene nucleotide sequence.

Fig. 1-a. Buffered Charcoal Yeast Extract (BCYE) agar plate showing *Legionella* colonies

Fig. 1-b. Magnification of *Legionella* colonies showing the characteristic structure of the colony /(gray colored with hallow zone around each colony).

Fig. 2. Gram stained film from presumptive *L. pneumophila* culture showing Gram negative non performing rods (bacilli), they range in size and shape from uniform small bacilli 0.5 by 2.0 µm.

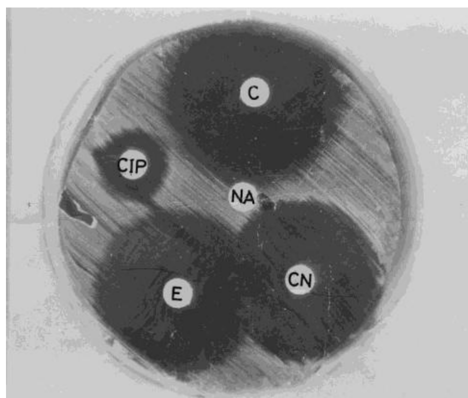

Fig. 3-a. Antibiotic sensitivity test for *Legionella* showing; sensitive to; Gentamicin (CN), Erythromycin (E) and choloramphenico (C); resistant to; Nalidixic acid (NA) and Ciprinol (CIP).

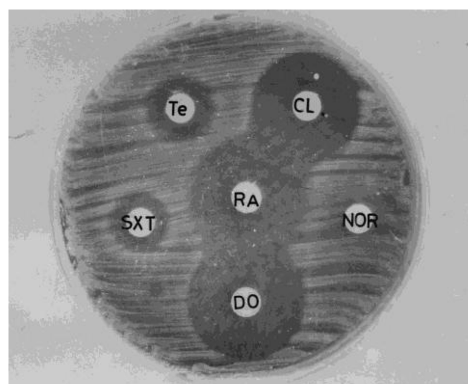

Fig. 3-b. Antibiotic sensitivity test for *Legionella*, it is sensitive to; Doxycycline hydrochloride (Do), Rifampicine (RA), and Cephalexin (CL), and resistant to; Salphamethoxazole trimethoprim (SXT), tetracycline (TE), and Noroxine (NOR).

Table 2. Strength of agglutination reaction test.

Sample	Number of sampled	Positive		Strength*			
		No.	%	1+	2+	3+	4+
Water	(`140)	85	60%	10	20	25	30
Swabs	(40)	30	75%	-	-	10	20
Patients	(12)	12	100%	-	-	8	4

1+ : Light agglutination with suspension 2+ : Heavy agglutination with suspension

3+ : Light agglutination with clear zone 4+ : Heavy agglutination with clear zone

Tables 3. *L. pneumophila* serotype by using latex agglutination test (serotype 1-14).

No. of specimens	Positive		Serotypes													
	No	%	1	2	3	4	5	6	7	8	9	10	11	12	13	14
140 water sample	85	60	5	6	4	7	20	-	8	5	10	2	2	7	6	3
40 Swabs	30	75	3	2	5	4	6	-	-	2	3	2	1	-	-	2
12 Patient spu-ta	12	100	-	2	1	1	-	-	2	1	1	2	1	-	1	-

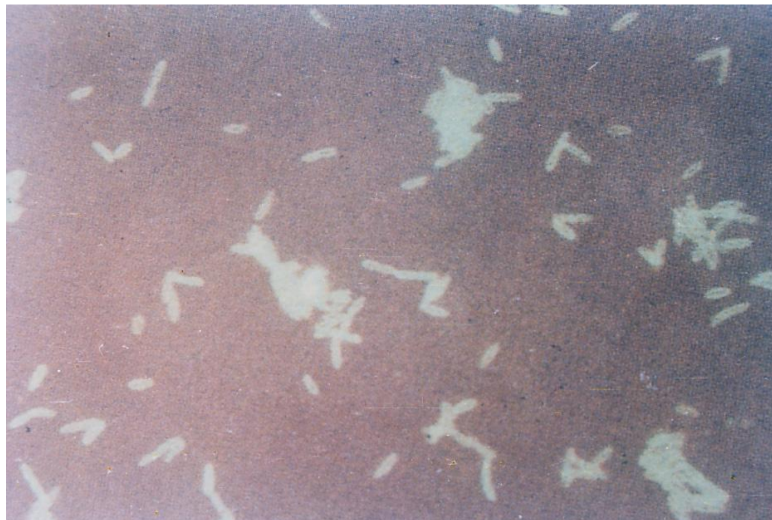

Fig. 4. DET using (FITC) labeled anti-*L. pneumophila* monoclonal antibody (mouse) the bacilli green fluorescence under the fluorescent microscope.

Table 4. Direct immunofluorescence test* for *L. pneumophila* In 192 samples from different sources.

Organism	Positive
L. pneumophila serotype	
1	19
2	10
3	5
4	8
5	6
6	8
7	13
8	6
9	8
10	8
11	9
12	5
13	3
14	2

* 140 water sample, 40 swabs & 12 patient sputa

The specific band of *L. pneumophila* was seen at 649 bp as shown in **Fig. (5)** and **Table (5). Ramirez *et al* (1996)** found that PCR was able to detect 15 of the 17 infected samples (88.2%), this indicate that PCR is a rapid, sensitive and specific test that may greatly simplify the diagnosis of *Legionella* spp. In another study, **Lu *et al* (1997)** reported that PCR method provided a quick and accurate way not only to detect *L. pneumophila* in the diagnosis of LD but also in the capture and suspected pathogen during epidemiologic investigation when LD outbreaks.

Disinfection procedures for *L. pneumophila*

We evaluate the efficacy of 4 disinfection modalities a controlled and comparative fashion: chlorine (4 to 6 mg/liter), heat (50 to 60ºC), ozone (1 to 2 mg/liter), and UV light (30,000µW-5/cm^2). Each disinfectant was tested under 3 conditions: (i) nonturbid water at 25ºC, (ii) turbid water at 25ºC and (iii) nonturbid water at 43ºC.

Our results show that all four methods were efficacious in eradicating *L. pneumophila* from the model plumbing system. The application of chlorine, ozone and UV light showed a 5 to 6 log decrease of *L. pneumophila* within 6 h of continuous disinfection. Heat disinfection eliminated all legionellae within 3 h of disinfection with chlorine, ozone and UV light. UV light produce a 5 log kill within 20 min, while, chlorine, ozone and heat required considerably more time to achieve the same degree of killing **(Fig. 6).** In this model, however, turbidity was not shown to impair the efficacy of any of the four disinfection methods **(Fig. 7).**

Higher water temperature (43ºC) enhanced the disinfecting efficacy of chlorine, whereas ozone and UV light were unaffected **(Fig. 8).** Enhanced efficacy of chlorine in killing *L. pneumophila* at higher temperature was also noted by **Kuchta *et al* (1983).** This may be a result of accelerated binding of chlorine to the cell surface **Walker *et al* (1995).** However, it should also noted that the addition of approximately 120% more chlorine was necessary at the higher temperature of 43ºC to overcome the thermal decomposition of the chlorine residual **(Fig. 9)**.

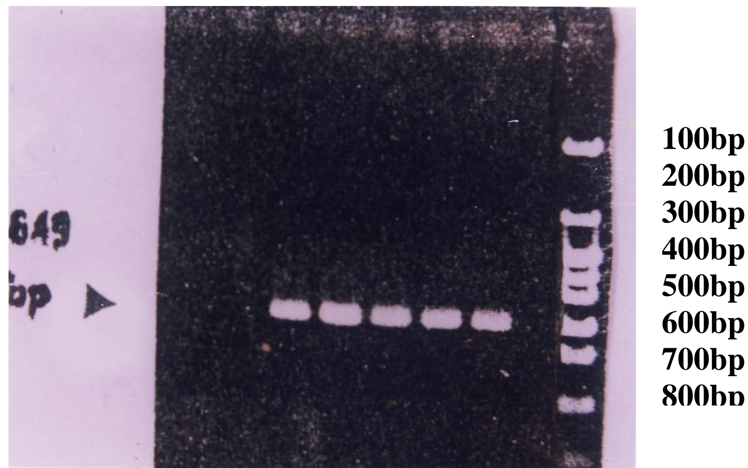

Fig. 5. Agarose (2%) gel electrophoresis of amplified DNA from contaminated samples performed with primers LmipL 710 and LmipR 1686 and with primers LmipL 920 and Limp R1548, pHY300 PLK and Hae III-digested pHY 300. 2PLK were used as molecular weight markers, the specific band for *L. pneumophila* was seen at 649 bp.

Table 5. Polymerase chain reaction on isolated colonies using the specific primer (LmipL 710 and Limp R 1686 and with primers Limp L 920 and Limp R 1548) as external and internal primers

Source of sample	Number of sample	Positive tests	
		No.	%
Water	140	90	64.3
Swabs	40	30	75
Sputum	12	10	83.3

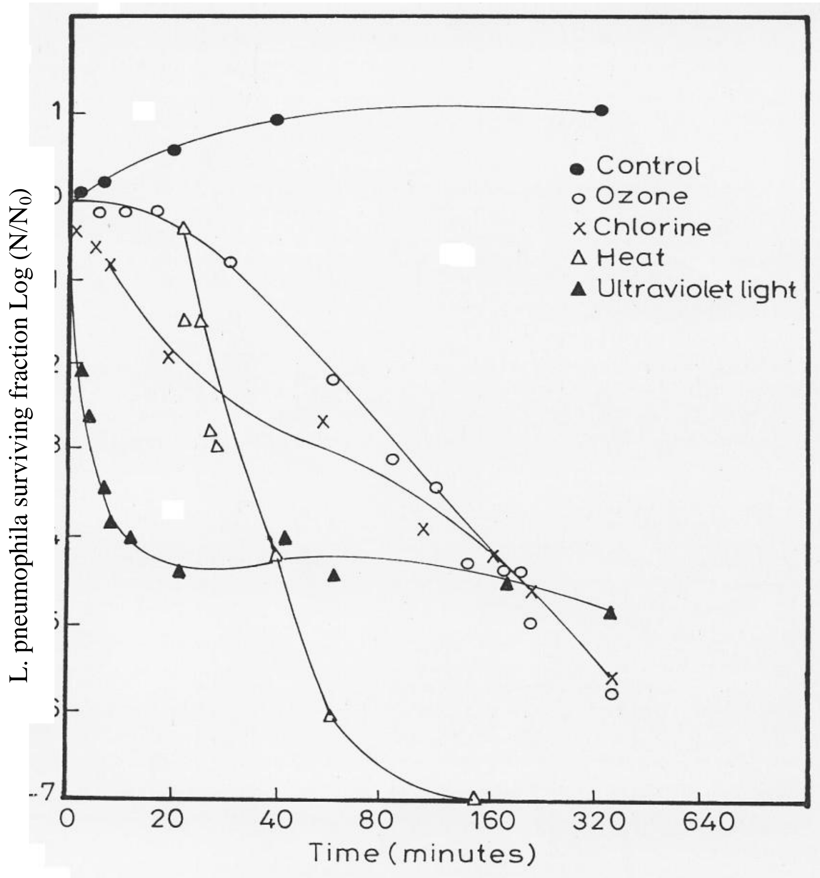

Fig. 6. The comparative efficacy of chlorine, ozone, heat, and UV light in eradicting *L. pneumophila* from a model plumbing system. Each disinfectant technique was evaluated individually in nonturbid water at 25˚C. Mean disinfectant levels were as follow : Chorine (4 to 6 mg/L), heat (50 to 60˚C) , ozone (1 to 2 mg/L) and UV light (30,000uW-s/cm^2). The control plot represents a fectant method. The plots are presented in the form log (N/No) vs. time (t), where N= *L. pneumophila* in CFU per milliliter at any time (t) and No = *L. pneumophila* in CFU per milliliter at t = 0

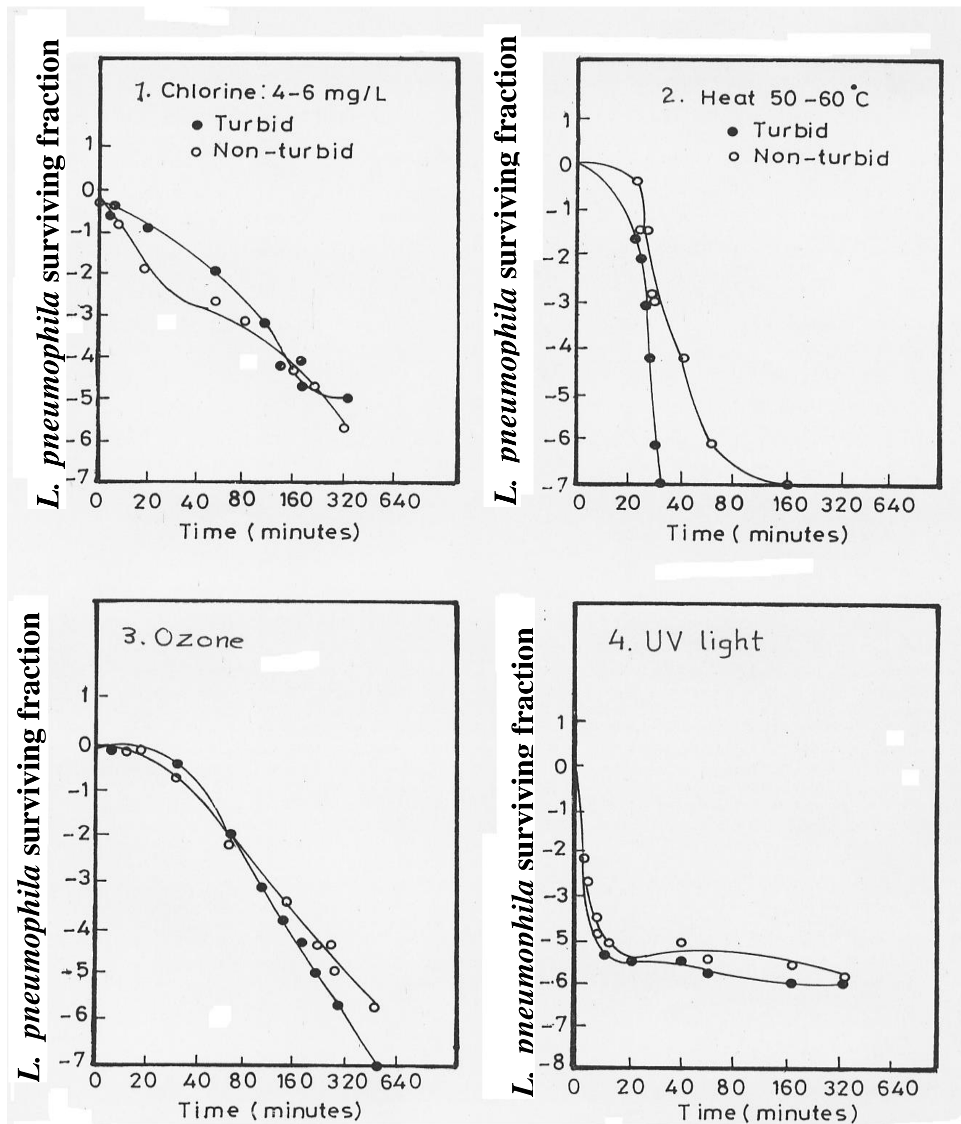

Fig. 7. Effect of turbidity on the efficacy of chlorine (1), heat (2), ozone (3) or UV light (4) Turbid water was prepared by making a 1:10 dilution from concentrated hot water tank effluent samples. This water was determined to have a suspended solids concentration of 4 to 5 mg/L. Tap water was used as a nonturbid medium. The plots are presented in the form log (N/No) vs. time (t), where N= *L. pneumophila* in CFU per milliliter at any time (t) and No= *L. pneumophila* in CFU per milliliter at t = 0.

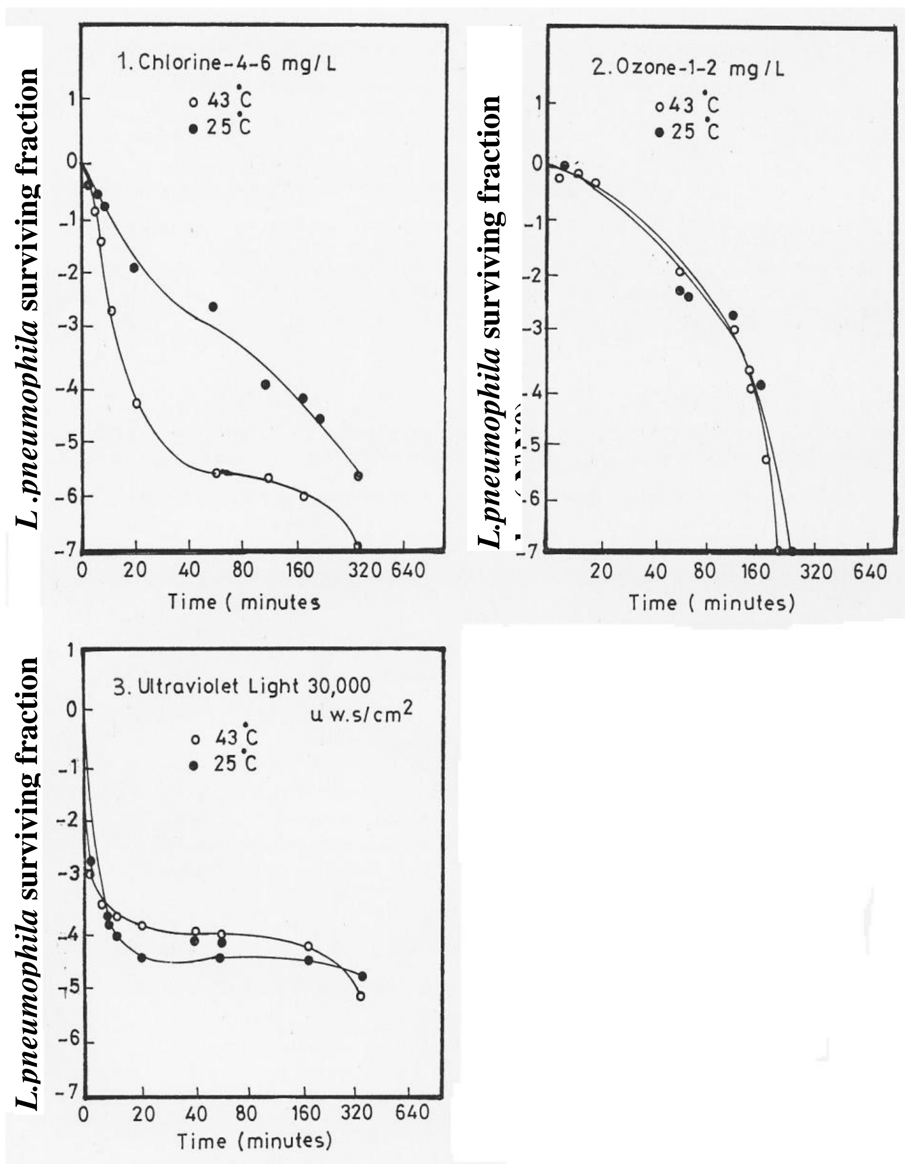

Fig. 8. The effect of temperature on the efficacy of chlorine (1), ozone (2), and UV (3). Increasing the water temperature (25,vs. 43˚C) enhanced the efficacy of chlorine, whereas ozone and UV light were unaffected. The plots are presented in the form log (N/No) vs. time (t), where N = *L. pneumophila* in CFU per milliliter at any time (t) and No = *L. pneumophila* in CFU per milliliter at t = 0.

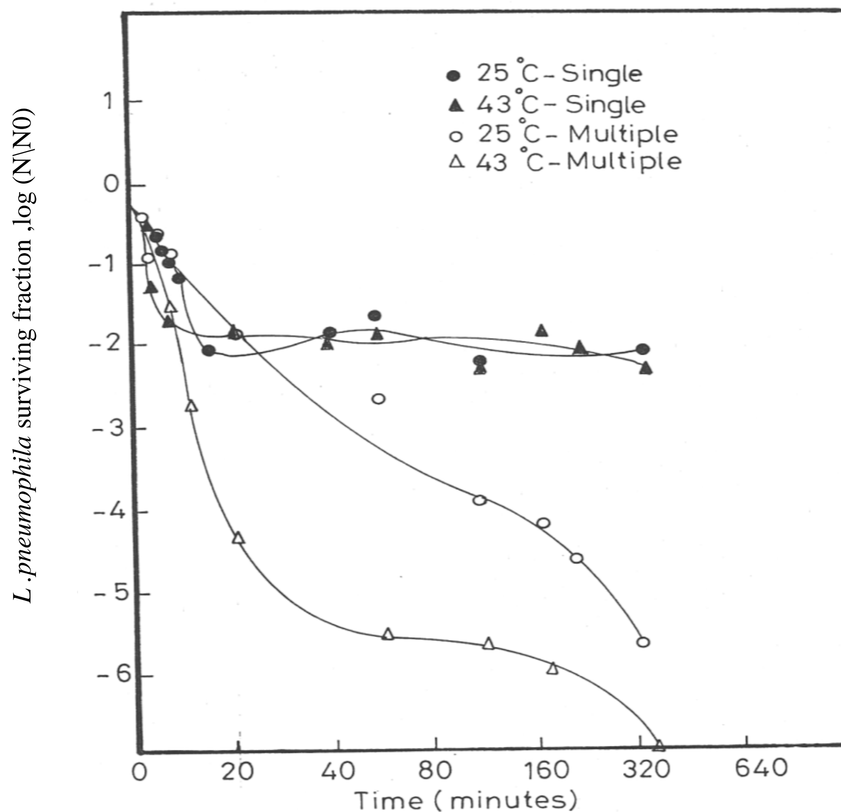

Fig. 9. The efficacy of chlorine disinfection is depended upon maintaining a residual chlorine conc. The plots designated (s) depict Legionella survival when chlorine was administered as a single injection of 8 ml. After 20 to 40 min. *L. pneumophila* numbers remained stationary because of diminishing chlorine residual concentration. The plots designated (m) depict a 5 to 6 log decrease of *L. pneumophila* when the chlorine residual concentration was maintained at 4 to 6 mg/L. by multiple addition. To maintain a chlorine residual of 4 to 6 mg/L for 6h, 18 ml of chlorine was necessary at 25˚C, whereas 40ml of chlorine was necessary at 43˚C. The plots are presented in the form log (N/No)vs. time (t) , where N= *L. pneumophila* in CFU per milliliter at any time (t) and No=Hyperchlorination at 4 to 6 mg/liter proved efficacious in suppressing *L. pneumophila* contamination **(Harley *et al* 1997)** but when the chlorine residual dropped below 4 mg/liter, cases of nosocomial legionellosis reappeared.

The application of heat (50 to 60ºC) eradicated *L. pneumophila* from the model system within 3 h. These results have been duplicated in hospital water systems where heat has been used as primary disinfection modality **Mietzner *et al* (1997).**

An ozone residual of 1 to 2 mg/liter was shown to effectively control *L. pneumophila* within this model system. Although one study of ozonation in a hospital was inconclusive, the data suggested that ozone could suppress *L. pneumophila* in a large water distribution system **(Zacheus and Martikainen 1996).**

The efficacy of UV light for eradication of *L. pneumophila* has been demonstrated *in vitro* **(Moreno *et al* 1997).** In our model system, *L. pneumophila* concentrations decreased by 4 to 5 logs with UV irradiation within 20 min, whereas chlorine and ozone required at least 3 h to achieve the same degree of killing (Fig. 6). UV light disinfection was not impaired by conditions of turbidity or increased temperature. From the results of this study, UV irradiations appear to have potential as a primary or supplemental in situ disinfectant method.

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دراسات عن التواجد، والتشخيص، والتحكم في بكتيريا الليجيونيلا بنيموفيلا في مصادر المياه المختلفة

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تهدف هذه الدراسة نحو معرفة أنسب وأدق الطرق المستخدمة في الكشف عن بكتيريا الليجيونيلا بنيموفيلا (المسببة لمرض إلا لتهاب الرئوي في الإنسان) وكذلك دراسة طرق القضاء عليها في مصادر المياه المختلفة بمستشفيات جامعة المنصورة وقد أجري هذا البحث علي 192 عينة ،منها 140 عينة مياه من مصادر مختلفة (دورات مياه المرضي بمركز جراحة الجهاز الهضمي ،ومركز الكلي، ووحدة الصدر بمستشفي المنصورة الجامعي وأجهزة التكييف المركزي)، 40 عينة قطالة (مسحة) من شبكة المياه الداخلية بالمستشفيات ، 12 عينة بصاق (8 عينات من مرضى مصابين بأمراض الكبد و4 عينات من مرضى الفشل الكلوي ، وتم زراعة كلي لهم) زوتم اختيار عدة طرق لزراعة وعزل بكتيريا الليجيونيلا بنيموفيلا علي المنبت الغذائي المكون من الخميرة والفحم الحيواني (Buffered charcoal yeast extract medium) الخاص بزراعة هذه البكتيريا ووجد أن الطريقة المقترحة بواسطة ريبيرو ومعاونيه سنة 1987 هي انسب طريقة وفيها يتم حجز البكتيريا علي غشاء الترشيح الخاص بالبكتيريا Millipore filter (0.20µ) ثم يتم إذابة هذا الغشاء ب 10 مللي ماء معقم باستخدام جهاز فورتكس وبتركيز هذا المعلق الذي يحتوي علي البكتيريا باستخدام جهاز الطرد المركزي ثم تزرع باستخدام (1ملليتر) علي الوسط الغذائي المشار إليه.

وقد أظهرت نتائج هذه الدراسة أن 148 عينة من بين العينات المختبرة البالغ عددها (192 عينة) تحتوي علي عزلات بكتيرية ذات خصائص مورفولوجية ومز رعيۃ وكيمو حيوية وفسيوليجية مطابقة لخواص بكتيريا الليجيونيلا بنيمو فيلا ، الأمر الذي يدل علي وجود هذه البكتيريا بمصادر المياه وبالعينات المأخوذة من المرضى ،كذلك وجد أيضا أن هذه البكتيريا مقاومة لكل من السيرونيول وحمض الناليديكسيك وسلفاميثوزول تريمثوبريم

والنوركسين والتتراسيكلين ولكنها حساسة لكل من الجنتاميسين والارثيروميسن والكلورامفينيكول والدوكس سيكلين والريفاميسين والسيفاليكسين.

وبعد إجراء إختبار التلبد (التخثر) وجد أن 107 عينة من 148 عينة مياه موجبة لهذا الاختبار، 30 من 40 عينة قطالة (75%) موجبة وجميع عينات المرضي كانت موجبة أي بنسبة (100%) وظهرت نتيجة التحليل المجهري والفلورسنتي المباشر لجميع هذه العينات (192 عينة) باستخدام 14 طراز سيرولوجي بكتيرية الليجيونيلا بنيموفيلا علي النحو التالي: 110عينة موجبة، 82 عينة سالبة. وباستخدام تقنية تفاعل البلمرة المتسلسل (PCR) للكشف عن الليجيونيلا بنيموفيلا وجد إن 130 عينة موجبة لهذا التحليل، 62 عينة سالبة. ولقد أسفرت النتائج إن تقنية PCR يمكن الاعتماد عليها في الكشف الدقيق عن هذا النوع من البكتيريا نظرا لحساسيتها العالية.

وأخيرا تم استخدام عدة طرق لمكافحة الليجيونيلا بنيموفيلا في مصادر المياه المختلفة بالمستشفيات منها الأوزون، الكلور، الأشعة فوق بنفسجية، والتسخين لدرجات الحرارة المرتفعة، ووجد أن كل من هذه الطرق منفردا يؤدي إلي اختفاء هذه البكتيرية الأمر الذي يوحي بقتلها وإبادتها وخاصة مع ارتفاع درجات الحرارة، إلا أن طريقة الكلور هي اقل هذه الطرق كفاءة في مقاومة هذه البكتيريا بالإضافة إلي أن إستمرار استخدامها لفترة زمنية طويلة قد يؤدي إلي تراكم بعض النواتج الثانوية غير المرغوب فيها في مصادر المياه.

ويتوقف اختيار الطريقة الصحيحة علي أساس طبيعة المكان والإمكانيات المتاحة بها.

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