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Legionella pneumophila Isolated from Cancer Patients and Hospital Environments

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ABSTRACT

A total of 360 samples (Clinical 271 and 89 environmental samples) were collected from the Oncology Center at Al-Sadr hospital in Basrah city southern of Iraq, during January- March, 2020. The clinical specimens included blood, urine and sputum, were taken from patients attending and /or admitting to the center. Meanwhile, the environmental samples were collected from air conditioners, hospital toilets and water.Three hundred isolates of presumptive Legionella sp. were identified using morphological characteristics, biochemical testing and one hundred were subjected for serotyping tests,

The morphological features of L. pneumophila on BCYE agar are all strains produce round, shiny and white colored colonies with a hardly obvious green at 3 days incubation.L. pneumophila also identified using biochemical tests, which include: catalase, oxidase, DNase, gelatin liquefaction, hippurate hydrolysis, urease, biofilm forming (tube and Congo red methods and tissue culture plate method), starch hydrolysis, citrate utilization, hemagglutination activity, protease production and lecithinase and lipase production. The serogroup of Legionella pneumophila was identified using HiLegionella Latex Test Kit. The results showed that 85 isolates were serogroup 1 and 15 isolates were serogroup 2-15. In addition to that nine types of antibiotics were used to determine the susceptibility of 93 isolates to resist them which including azithromycin 15 µg, cefotaxime 30µg, ciprofloxacin 5µg, doxycycline 30 µg, erythromycin 15 µg, levofloxacin 5µg, ofloxacin 10 µg, norfloxacin 10 µg and Rifampicin 5 µg. Furthermore, ten of 23 isolates resistance to antibiotics was subjected to the test of minimum inhibitory concentrations using MIC strips which including azithromycin, cefotaxime, ciprofloxacin and levofloxacin.

Keywords: *Antibiotic susceptibility, Cancer center, Legionella pneumophila, MIC*

INTRODUCTION

Cancer is a condition in which a collection of aberrant cells multiplies uncontrollably while defying the usual laws of cell division (Bekele, 2022). Normal cells are persistently subject to signals that specify in what order the cell should divide, develop into another cell, or die. Yet, cancer cells can become somewhat autonomous from these signals, leading to uncontrolled growth and proliferation that can be lethal if allowed to continue and spread; In reality, tumor metastasis a process known as tumor spread causes about 90% of cancer-related fatalities instances (Baloch et al., 2022; Heald, 2021). Legionella microorganism is ubiquitous and found worldwide naturally in rivers, streams, springs of hot water, swimming pools, tanks, water piping networks, cooling tower and conditioning systems (Khaledi et al., 2018). This bacterium causes sporadic and epidemic cases of community-acquired pneumonia (CAP) in healthy and immunocompromised from hospital or community settings (Bagheri et al., 2021).

The majority of the 65 recognized species of legionellae are expected to be able to inflict human disease under the right circumstances, however only around half of these species have been linked to legionellosis (Chauhan and Shames, 2021). Of the 15 serogroups of this species, Legionella pneumophila serogroup 1 is responsible for 84% of confirmed cases of legionellosis and is thought to be the cause of about 91% of all known community cases (Yu et al., 2002). The Legionella related illnesses are collectively known as legionellosis, and they often manifest as two different clinical entities. The pneumonic variant of the deadly multisystem illness legionellosis, which includes pneumonia, is known as Legionnaires disease (National Academies of Sciences, 2020). Legionella has the propensity to proliferate in human alveolar macrophages due to its natural capacity to do so within several protozoa. Understanding how L. pneumophila interacts with eukaryotic cells will help us better understand how the bacterium causes disease.

Given the lack of in-depth studies on the bacteria associated with cancerous infections and the excessive use of antibiotics in a random manner especially in Iraq.

This study was designed and aimed for the isolation and identification of Legionella sp. depending on their morphological features, biochemical and serological profiles from cancer patients and hospital environments. In addition, the use of different antibiotics may be helps to limit the spread of these bacteria and does not affect the patient's health.

MATERIALS AND METHODS

Sampling

A total of 360 samples were collected from the Oncology Center at Al-Sadr hospital in Basrah city. Clinical samples 271 and 89 environmental samples, during January- March, 2020. The clinical specimens included blood, urine and sputum, were taken from patients attending and /or admitting to the center whom suffering from different types of cancer as breast, lung, osteosarcoma, prostate, ovary, pancreatic, stomach, colorectal, liver, bladder, uterus, and kidney. Meanwhile, the environmental samples were collected from air conditioners, hospital toilets and water (Table 1).

Isolation and identification of bacteria

The bacteria were isolated using three different types of media, including nutrient agar, blood agar, and buffered charcoal yeast extract (BCYE) agar. The colonies were appeared after incubating for 24-48 h. at 37 °C, and subjected for morphological, serological and biochemical tests as gram stain, oxidase, catalase, motility, DNase, hippurate, nitrate reduction, urease, starch hydrolysis, gelatin liquefaction, lecithinase and lipase production, hemagglutination activity, tolerance of different levels of pH, effect of temperature on bacterial growth, tolerance to salinity, and detection of biofilm formation (tube method, Congo red agar, tissue culture plate). In addition to that the bacteria were tested for their antimicrobial susceptibility assay using disk diffusion method (Liofilchem, Italy) (Table 2), and MIC strip (Hi Media, India) (Table 3).

TABLE 1: Sources and number of clinical and environmental specimens.

Samples	No. of Specimens
Clinical specimens	271
Blood	213
Urine	19
Sputum	39
Environmental specimens	89
Bathrooms	23
air-conditioning (Filters)	9
Air samples	24
Water samples (Faucet filters)	33

TABLE 2: Antibiotic discs with concentrations and codes.

No.	Antibiotics	Concentration	Code
1	Azithromycin	15 µg	AZM
2	Rifampicin	5 µg	RD5
3	Ciprofloxacin	5 µg	CIP
4	Norfloxacin	10 µg	NOR
5	cefotaxime	30 µg	CTX
6	Levofloxacin	5 µg	LEV
7	Doxycycline	30 µg	DXT
8	Ofloxacin	10 µg	OFX
9	Erythromycis	15 µg	E15

TABLE 3: MIC Strip with concentrations and codes.

No.	Antibiotics	Concentration	Code
1	Azithromycin	0.016-256 mcg\ml	AZI
2	Ciprofloxacin	0.016-256 mcg\ml	CIH
3	Cefotaxime	0.002-32 mcg\ml	CTX
4	Levofloxacin	0.002-32 mcg\ml	LEV

RESULTS

Distribution of cancer cases according to age groups, gender and type of cancer

Three age groups (Fig. 1) were studied, less than 30 years old (80 samples, 29.5% of the total patients). 30-60 years old were 100 samples (36.9%). And finally more than 60 years old were 91 samples (33.6%). According to gender, the number of male patients was112 (41%) and the number of female patients was159 (59%). The results of the statistical analysis showed significant differences ($p < 0.01$) between male and female patients. As for the types of cancer, the current study recorded 137(50.5%) cases of leukemia, lymphoma 28 (10.3%), and 106 (39.1%) of solid tumors**.**

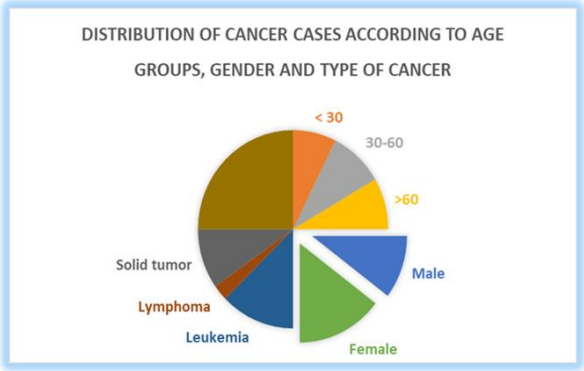

FIGURE 1: Distribution of cancer cases according to age groups, gender and type of cancer.

From 360 samples, 300 isolates as presumptive Legionella sp. are isolated from blood samples (185 isolates), urine samples (24 isolates), sputum samples (33 isolates), and air samples (35 isolates) and from water samples (13 isolates). All these isolates are identified using morphological characteristics, biochemical tests and serotyping.

Morphological properties:

On BCYE agar, isolates are produced round, shiny and white colored colonies with a hardly obvious green after 24-48 h. of incubation. Colonies will be larger after 5 to 7 days of incubation, becoming more distinctly green or gray-blue flat, dull, and opaque in appearance ,(Fig2).

FIGURE 2: Legionella ,BCYE agar

Identification and Characterization of Legionella sp. The biochemical tests are conducted for all 300 isolates (Table 4), there are a variation in the respond of bacteria to these tests and only 100 isolates seem to fit with presumptive Legionella sp.

TABLE 4: biochemical tests for all isolates bacteria.

Sample	Catalase		Oxidase		DNase		Gelatin		Hippurate		Urease		Starch	
	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Blood	178	7	146	39	185	-	157	28	165	20	69	116	8	177
Urine	19	5	19	5	24	-	20	4	20	4	12	12	1	23
Sputum	39	4	38	5	40	3	34	9	34	9	16	27	4	39
Air	33	2	32	3	32	3	28	7	31	4	21	14	4	31
Water	12	1	9	4	12	1	8	5	8	5	6	7	2	11
Total	281	19	244	56	293	7	247	53	260	40	124	176	19	281

Samples	Motility		Nitrate		Citrate		Protease		Lecithinase and Lipase		Hemagglutination		
	+	-	+	-	+	-	+	-	+	-	+4	+3	+2
Blood	183	2	185		185		185	-	152	33	67	107	11
Urine	24	-	24		-	24	24	-	20	4	11	11	2
Sputum	43	-	43		43		34	9	34	9	13	23	7
Air	35	-	2	33	3	32	35	-	27	8	14	19	2
Water	13	-	1	12	1	12	12	1	10	3	6	5	2
Total	298	2	3	297	4	296	290	10	243	57	111	165	24

Identification of Biofilm forming pathogenic bacteria

Tube method: Tube method was used as a qualitative assay for detection the biofilm forming bacteria isolated from cancer patients, the results showed that 4 (26.6%) isolates were strong biofilm former, 3 (20%) moderate, and 8 (53.3%) weak or non-biofilm forming bacteria.

Congo red agar method: isolates were cultured on Congo red agar for detection the biofilm forming bacteria. 105 isolates (35%) were negative and 195 isolates forming biofilm (65%) (Table 5) (Fig 3)

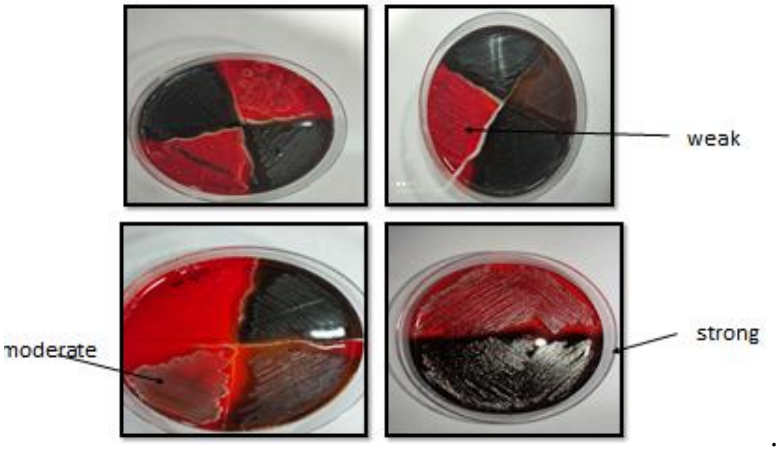

FIGURE 3: Biofilm forming on Congo red agar.

Legionella serogroup:

According to the results obtained from the biochemical tests, only 100 isolates matched with the biochemical features of Legionella, which were subjected to the serological tests. 85 isolates belong to sero group 1 and the rest 15 isolates are sero group 2-15 (Table 5).

TABLE 5: Biofilm forming using Congo red method and serological test.

Sample	Biofilm forming		Serology test	
	+	-	Sero group 1	Sero group 2-15
Blood	125	60	25	2
Urine	13	11	23	1
Sputum	30	13	27	3
Air	21	14	4	4
Water	6	7	6	5

Tissue culture plate method (TCP): The 100 isolates are subjected to TCP test. 35 isolates (35.0%) showed strong biofilm production, 32 (32.0%) has moderate production, and 33 (33.0%) has low production.

TABLE 6: The results of tissue culture method.

Mean OD values	Frequency	Isolates No.	Percent
>0.240	Strong	35	35.40
0.120-0.240	Moderate	32	32.0
<0.120	Weak	33	33.0

Antibiotic susceptibility (Disk diffusion method):

Nine types of antibiotics are used to determine the susceptibility of 93 isolates. The inhibition zones of antibiotics are compared to CLSI and the results showed that all isolates are sensitive

to LEV5, CTX30, OFX, CIP5 and RD5, while the rest shows variable sensitivity as 92 isolates sensitive to NOR10 (98.9%) > 90(96.8%) to DXT30 > 85 (91.4%) to AZM15 and 82 (88.1%) to E15. This test showed significant difference P≤0. 01.

Antibiotic susceptibility (MIC Strip):

Four strips are used in the current study. Ten isolates that are resistance to antibiotics are used in this test. Three isolates are resistance to CTX, one isolate is obtained from blood, 1 from sputum and 1 from water samples,(Fig 4).

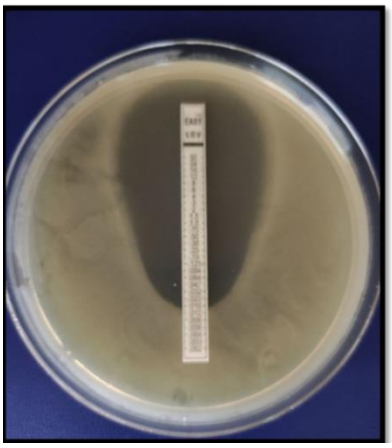

FIGURE 4: Antimicrobial susceptibility test for MIC strips.

DISCUSSION

Hospital acquired infection (HAI) is a significant issue everywhere. Fighting HAI requires a thorough understanding of bacterial etiology patterns and antibiotic susceptibility. Nosocomial infections or healthcare-associated infections in immunocompromised cancer patients may be caused by hospital or healthcare facility supplies and equipment that are frequently contaminated with bacterial strains that can spread to immunocompromised individuals (Abdallah et al., 2008b).

In order to isolate the accurate bacteria three media are used as BCYE, blood agar and nutrient agar. The colonies are appeared on the BCYE agar shinny gray to bluish gray, white-gray, convex with smooth edges. Young colonies' middle region seemed to be light gray, granular-like, with a glass background, while its periphery was bright pink or blue. This result corresponds to Ditommaso et al. (2022); Mousavi et al. (2022); Niculita-Hirzel et al. (2022) and Barzegar et al. (2022), which are used the same medium. The purpose of using this medium is that contains many components that support the growth of these bacteria. (Lorry and Rubin, 2018; Priya et al., 2022; Bhatt et al., 2022).

In the present study deoxyribonuclease an extraenzyme were detected and about 97.7% of isolates are positive and only 2.3% are negative for this test. This result in accordance with other studies (Mu'azu etal., 2021 ; Zonta et al., 2021 and Prommachote et al., 2022). The ability of produce gelatinase are investigated and found that 82% of isolates are positive, meanwhile 18% are negative. This finding is similar with those of Jiang et al. (2022), Ghernaout et al. (2022), and Mahdi et al. (2021).

The results of a hippurate hydrolysis experiment revealed that 86.7% of isolates are positive and 13.3% are negative, this enzyme hydrolyzing hippurate into glycine and benzoic acid. More than half of tested isolates have a negative reaction for urease (58.7%), and this including also starch hydrolysis which the negative isolates reached 93.7%. The results of the motility test showed positive tests for 99.3% of isolates and two negative isolates (0.7%). This test is one of the important diagnostic tests for Legionella, and the positive result is an indication of the motility of this bacteria. For infection to proceed, Extracellular proteases are used as a first line of defense when the pathogen escapes its protective intracellular niche and is made vulnerable to the host immune response. There were signed of L. pneumophila metallo protease would have the capacity to break serum proteins (Scheithauer et al., 2021; Figueroa et al., 2021), and the results of the present study indicate the percentage of negative isolates are 3.3% and the percentage of positive isolates are 96.7%; significant differences were recorded P≤0.01 .

The ability of bacteria to reduce nitrate have monitored about 99% of isolates are negative and this finding were in agreement with published researches (Jiang et al., 2022; Ghomimaghsad et al., 2020). Extracellular pathogenicity of L. pneumophila is characterized by significant tissue destruction, including extracellular matrix degradation and localized septal disruption and secreted effector molecules are essential for this pathogenicity (Talapko et al., 2022). The pulmonary and extrapulmonary abnormalities that come along with Legionnaires' illness have been theorized to be caused, at least in part, by Legionella pneumophila producing extracellular enzymes or poisons. It has been demonstrated that L. pneumophila can produce a variety of

toxins and enzymes that could be hazardous. Exotoxins having cytotoxic and hemolytic properties have been identified (Arslan-Aydodu and Kimiran, 2018). Bacterial adhesion to the host mucosa is caused by pili, which are organelles. On the surfaces of L. pneumophila, Stone and Abu Kwaik demonstrated two different forms of pili. One of these pilus varieties was the type IV pilus, which may cling to host cells in mammals without complementation. The result of The importance of hemagglutination activity and, thus, the existence of type IV fimbria as a virulence factor in revealing bacterial pathogenicity is emphasized by the hemagglutination assays (Baine et al., 1979). The results of the current study are clarified as follows: 37% ++++, 55% +++, and 8% for the ++ group. The statistical analysis' findings demonstrated that there were substantial disparities between the three groups. The results were all positive, with the exception of taking into account the period of blood agglutination, Wwhich agree with Arslan-Aydodu and Kimiran (2018). The current study results of Legionella serogroup tests of the statistical analysis of the serogroup 1 test showed that 85% of isolates are positive, meanwhile 15% returned to serogroup 2-15. This result agrees with Cocuzza et al. (2021) and Tata et al. (2022) who reported that the primary method for diagnosing Legionnaires' illness is the finding of Legionella pneumophila serogroup 1 antigen in urine. For patients with legionellosis, however, there have been improvements in detection tests. The results of current study of antimicrobial susceptibility test for 93 isolates, showed All isolates are 100% sensitive to LEV5, CIP5, CTX30 and OFX, as well as results showed that most isolates were susceptible to DXT30, NOR10, RD (96.8%, 98.9% and 99.9%) respectively. However, the results were shown for two antibiotics, represented by the least sensitive bacterial proportions AZM 15, E15 (84%, 87.1 %) respectively. This result corresponds to De Giglio et al. (2015) and Portal et al. (2021) who pointed that the following antimicrobial compounds were evaluated against bacterial strains using standard laboratory powders, azithromycin erythromycin, ciprofloxacin, levofloxacin, rifampicin, doxycycline, and tigecycline. Sreenath et al. (2019)pointed that rifampicin was the most potent drug followed by levofloxacin, while doxycycline and tetracycline were found to be the less active agents.

MIC Test strips are a type of quantitative assay for measuring out the minimum inhibitory concentration (MIC) of antibiotics against bacteria, which can be used to determine the right course of treatment for a patient and spot patterns of resistance. This method has been used on some samples that showed resistance to the antibiotics used in the previously mentioned method. The first antibiotic, CPH, had an effect on all tested samples, and the second and third antibiotics, AZI (100%) and LEV (100%), were both effective with all samples under study. As for the fourth antibiotic, CTX, it was positive in all samples except for three, which were negative. With the appearance of a significant difference ($P \leq 0.01$), the study's findings agreed with Al-Matawah et al. (2012), who referred to the plates were then incubated at 35 C for two days with enhanced humidity after the E-test strip had been put to the swabbed surface. In order to know the ability of L. pneumophila form biofilms, to investigate biofilms, three methods were used, first the tube method, we found that 26.6% of the isolates were strong biofilm formers, 20% were acceptable, and 53.3% were weak or non-biofilm formation bacteria. This result agrees with (Hussein et al. (2021) and Tahaei et al. (2021). Second method is Congo red agar, the results showed about 65% of isolates have the ability to form biofilm and 35% of isolates not forming, these results matche with Kaiser et al. (2013) and Jabir (2022) who indicated that the creation of rough black colonies by biofilm forming strains was utilized to distinguish them from pink colored colonies produced by non- biofilm forming isolates. And third the tissue culture plate method, the microtiter plate (96 well plates) assay detects biofilm formation by allowing the observation of bacterial adhesion to an abiotic surface (Coffey and Anderson, 2014). Although, greater specificity for the Congo red technique, while the MTP method has higher quantification and sensitivity.

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