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Uncovering the Hidden Dangers: Isolation and Identification of *Legionella pneumophila* in Air Conditioning Systems Across Egypt

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Abstract: The goal of this investigation was to isolate and find out ***Legionella pneumophila*** isolates and to assess their antibiotic susceptibility from various air conditioning units. From February 2022 throughout a two-year span, a number of 600 swabs and water samples were collected from air conditioners, chillers, and cooling towers. Water samples were treated with acid, concentrated, and cultured on buffered charcoal yeast extract (BCYE) agar. Identification was subsequently verified by biochemical tests, Real-Time PCR, and 16S rRNA sequencing. Antibiotic susceptibility was done using the Kirby-Bauer disc diffusion method. In the analysis of 600 samples, four (0.7%) tested positive for *Legionella spp.* using culture methods, which were further confirmed by Real-Time PCR; additionally, two samples were validated through 16S rRNA sequencing. The isolates exhibited positive results for oxidase, catalase, gelatin degradation, and nitrate reduction tests, while tests for urease activity returned negative results. Isolates of ***L. pneumophila*** displayed sensitivity to various antibiotics. The presence of pathogenic and potentially lethal ***L. pneumophila*** infections can be linked to air conditioning systems. Continuous monitoring of these appliances is essential to prevent the proliferation of this bacterium and associated health risks.

Keywords: Egypt; Air conditioning; ***L. pneumophila***; Real-Time PCR; 16S rRNA; Antibiotic susceptibility.

1. INTRODUCTION

Legionellosis encompasses various diseases caused by Legionella bacteria, including the severe form known as Legionnaires' disease and the milder Pontiac fever. Notably, more than 90% of cases of Legionnaires' disease cases are caused by *L. pneumophila* diagnosed worldwide. In Europe, serogroup 1 of *L. pneumophila* accounts for about 80% of human cases [1]. Epidemiological studies estimate the incidence of Legionnaires' disease globally to range between 0.5% and 10% among cases of community-acquired pneumonia [2].

The first isolation of Legionella occurred over 50 years ago from the blood of a soldier, highlighting the bacteria's presence in water environments and its significance as a human pathogen [3]. The association between *L. pneumophila* and human infection was formally documented in 1977 [4]. This bacterium can thrive in both natural and human-made aquatic environments, including lakes, streams, air-conditioners, cooling towers, fountains, and spa baths [5]. Transmission to humans typically occurs through aerosolized contaminated water from environmental or building water systems [6].

Clinical manifestations of Legionnaires' disease resemble those of other forms of pneumonia. The lack of distinctive symptoms underscores the importance of microbiological tests for accurate diagnosis. Most Legionnaires' disease cases are sporadic, with over 70% being community-acquired, although some instances are linked to travel or healthcare facilities [1].

Risk factors for Legionnaires' disease increase with age, particularly affecting individuals over 45, smokers, heavy drinkers, and those with chronic respiratory or kidney diseases, diabetes, lung and heart diseases, or compromised immune systems [7]. Studies have also suggested a correlation between the incidence of Legionnaires' disease and certain weather conditions, including warmer temperatures, higher humidity, and increased rainfall [8]. The global

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incidence of Legionnaires' disease remains largely unknown due to underdiagnosis and underreporting, compounded by variations in awareness, diagnostic methods, and investigation efforts across different countries [9].

Diagnosis of legionellosis relies on the combination of clinical and radiological signs alongside laboratory tests. These diagnostic tests are not routinely conducted in clinical microbiology laboratories; hence, they must be specially requested, utilizing methods such as the urine antigen test (UAT), polymerase chain reaction (PCR), and culture confirmation [10].

L. pneumophila is known for being a fastidious bacterium. Cells are characterized as weakly stained, thin, and somewhat pleomorphic Gram-negative bacilli, ranging from 2 to 20 µm in size, with some forming long filamentous structures [11]. *L. pneumophila* is ubiquitous and replicates inside free-living eukaryotic phagotrophs, mostly amoebae, including *Acanthamoeba castellanii, Acanthamoeba polyphaga, Dictyostelium discoideum*, and Naegleria spp. [11].

Treatment for legionellosis involves a combination of effective antibiotics tailored to the severity of the disease and individual patient factors. Optimizing patient outcomes requires early diagnosis and timely treatment [12]. However, limited information exists about side effects, complications, the period of antibiotic treatment, and how these relate to clinical outcomes.

This research accentuates detecting the presence of *L. pneumophila* in evaporative coolers and air conditioners from various locations. It aims to evaluate the proficiency of these environmental isolates for making biofilms as well as to test the inhibitory effects of natural products on biofilm formation.

2. METHODS

2.1. Collection of Samples

Over two years, commencing in February 2022, a number of 600 swabs and water samples were taken from different sites including air conditioners, chillers, and cooling towers during different seasons. Samples were obtained from distinct locations across three governorates in Egypt: Alexandria, Cairo, and Giza. Samples were collected in a sterile glass-stoppered bottle, it was then sterilized for 2h at 180ºC. Sterile cotton swabs were utilized to swab the filters of the air conditioners. Multiple samples were taken from the same location when appropriate. Following collection, samples were preserved in an icepack and transferred without delay to be treated for recovery of the isolates within 24 hours.

2.2. Sample Treatment

To inhibit the flourishing of unwanted organisms and to enhance the isolation of *L. pneumophila*, water samples were treated with a (1:10) KCl-HCl solution (pH 2.2). The mixture was agitated and then incubated at 25 ºC for 4 minutes [13].

2.3. Sample Concentration and Subculturing

Concentrating the diluted water samples was achieved by centrifuging for 10 minutes at 3000 rpm. The supernatant was thrown away., and the sediment was aseptically transferred for culturing on Legionella isolation media. Alternatively, filtration was carried out with nitrocellulose membranes with pore sizes of 0.45 µm. Direct inoculation of swab samples onto BCYE agar supplemented with BCYE Growth Supplement was done (HIMEDIA), containing ACES (N-(2-acetamido)-2-aminoethane sulfonic acid) buffer, potassium hydroxide, ferric pyrophosphate, L-cysteine hydrochloride, alpha-ketoglutarate, and distilled water. GVPC Supplement (glycine, vancomycin, polymyxin B, and cycloheximide) (Sigma, USA) was also added. The culture media were incubated at 37ºC and examined daily for up to 10 days for colonies exhibiting grayish-white, shiny appearances with smooth surfaces [14,15].

2.4. Identification of *L. pneumophila*

Suspected *L. pneumophila* colonies were examined microscopically to reveal their thin, weakly stained filamentous Gram-negative rods. The suspected colonies were subsequently subcultured on BCYE agar with and without L-cysteine, in addition to non-selective media including MacConkey agar for verification. Various biochemical tests were conducted to identify *L. pneumophila*, including the urease test, catalase test (using 3% hydrogen peroxide), oxidase test (using oxidase discs soaked with N, N, N, N-tetramethyl-P-phenylenediamine dihydrochloride), nitrate reduction test, and gelatin liquefaction test using 1% gelatin agar stab [16,17].

2.5. Molecular Identification of *L. pneumophila*

2.5.1. Real-Time PCR Identification

Real-time PCR was carried out using the microproof® Legionella Quantification LyoKit. For qualitative detection, results from channels FAM, VIC/HEX, and ROX (Legionella) were compared to channel Cy5 (Internal Control) for each sample.

2.5.2. 16S rRNA Gene Sequence Identification

Purification of whole genomic DNA was done using the E.Z.N.A.® Bacterial DNA Mini Kit (D3350-00S, OMEGA BIO-TEK, USA) following the manufacturer's instructions. PCR amplification was carried out using a specific primer pair for the

Legionella-specific 16S rRNA gene, as outlined in Table 1. DreamTaq Green PCR Master Mix (2X) (K1081, Thermo Fisher, USA) was used for particular gene amplification to comply with the manufacturer's protocol, employing the Creacon (Holland, Inc) Polymerase Chain Reaction (PCR) system cycler. The 16S rRNA served as a molecular identification marker, employing universal primers [18]. PCR products were analyzed using a 1.5% agarose gel stained with Ethidium bromide for 30 minutes, after which a 20-minute destaining period in distilled water.

2.6. Antibiotic Profile Study on *L. pneumophila*

Antibiotic sensitivity was conducted on *L. pneumophila* using the Kirby-Bauer disc diffusion method against the following antibiotics: vancomycin, rifampin, clarithromycin, meropenem, imipenem, erythromycin, clindamycin, tetracycline, levofloxacin, doxycycline, and ciprofloxacin. After adjusting the turbidity of the bacterial suspension at 0.5 McFarland and resulted in a suspension containing 1.5 x 10^8 CFU/ml with saline. Plates of BCYE media were prepared and allowed to dry before being inoculated with a bacterial suspension, which was evenly distributed using a sterile cotton swab and rotated several times. The plates were left to dry for 2 to 3 minutes. Subsequently, antibiotic discs were applied to the surface of the inoculated medium using sterile forceps and incubated at 37°C for 2 d. After incubation, the zones of inhibition were measured (mm) which reflect the activity of the antibiotics [19].

Table 1. 16S rRNA Primer sequences used in this study.

Gene name	Primer name	Sequence of the primer	Source
16s rRNA	16s rRNA F	AGGGTTGATAGGTTAAGAGC	[18]
	16s rRNA R	CCAACAGCTAGTTGACATCG	

**(F) Forward and (R) Reverse

3. RESULTS

3.1. Identification of *L. pneumophila*

A total of 20 isolates were retrieved, comprising 5 from hospital air conditioners, 3 from the University's air conditioners, 2 from residential unit's air conditioners, 3 from restaurant air conditioners, 5 from chillers, and 2 from cooling towers (table 2). These isolates were suspected to be *L. pneumophila* based on their morphology as pleomorphic Gram-negative bacilli, observed after subculturing on BCYE agar supplemented with L-cysteine; no growth was noted in the absence of L-cysteine (Figure 1).

The *L. pneumophila* isolates demonstrated no growth on non-selective media, such as MacConkey agar. Biochemical tests revealed that the isolates were gelatin liquefaction positive, catalase positive, nitrate reduction positive, oxidase positive, and urease negative (Figure 2 and Table 2).

Figure 1. Colony morphology of *L. pneumophila* on BCYE media showed gray-white, circular with a textured, cut-glass appearance.

Table 2. Suspected colonies for tentative identification of *L. pneumophila* isolates.

Suspected colony number	Source (air conditioners, Chillers and Cooling towers)	Growth on BCYE media	Growth on MAC media	Oxidase test	Urease test	Nitrate reduction test	Gelatin liquefaction test	Catalase test
Isolate 1	Hospital	+	+					
Isolate 2	University	+	+					
Isolate 3	Restaurant	+	+			NA (not legionella species)		
Isolate 4	Hospital	+	+					
Isolate 5	Chiller	+	+					
Isolate 6	University	+	-	-				
Isolate 7	Chillers	+	-	-		NA (not legionella species)		
Isolate 8	Hospital	+	-	-				
Isolate 9	Chiller	+	-	+	+			
Isolate 10	Cooling tower	+	-	+	+			
Isolate 11	Residential unit	+	-	+	+	NA (not legionella species)		
Isolate 12	Chiller	+	-	+	+			
Isolate 13	Cooling tower	+	-	+	-	-	NA (not legionella species)	
Isolate 14	Restaurant	+	-	+	-	-		
Isolate 15	Restaurant	+	-	+	-	+	-	NA
Isolate 16	Chiller	+	-	+	-	+	-	
Isolate 17	Hospital	+	-	+	-	+	+	+
Isolate 18	Residential unit	+	-	+	-	+	+	+
Isolate 19	University	+	-	+	-	+	+	+
Isolate 20	Hospital	+	-	+	-	+	+	+

**NA Not applicable

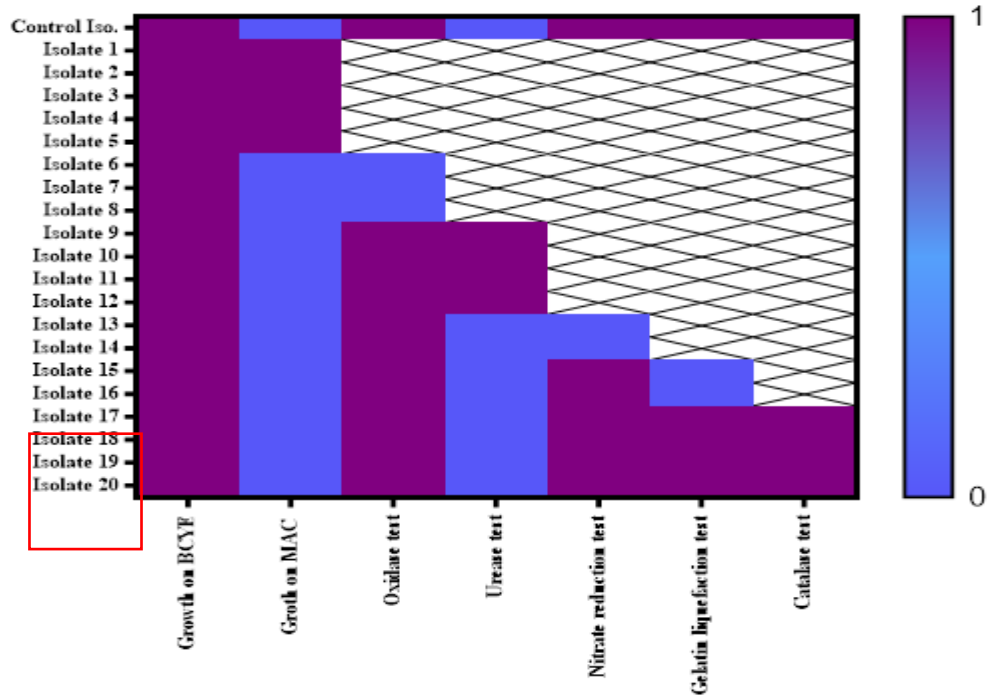

Figure 2. Suspected colonies and detected *L. pneumophila* isolates.

3.2. Real-Time PCR Identification

Four of the isolates showed positive results for *L. pneumophila* using the Real-Time PCR method (Table 3 and Figure 3).

3.3. Identification of *L. pneumophila* using 16S rRNA Gene Sequence

The phenotypic identification was confirmed by the gene sequences of 16S rRNA. The obtained results showed that the partial 16S rRNA gene sequences of both isolates with Accession numbers (PP417721) and (PP417722) are matched with the sequence of *L. pneumophila* AIRBIOTA-BSP3 and GC-A13 isolates respectively listed in BLAST (Basic local alignment search tool) of NCBI (National center of biotechnology information) data base with identity reached 100% and 93.71% respectively as shown in table (4). Thus, both isolates were identified as isolates of *L. pneumophila.* And the phylogenetic trees as shown in (Figures 4 and 5) respectively.

Table 3. Real-time PCR outcomes of L. pneumophila isolates using quantification cycle (Cq).

Well	Fluorophore	Sample Name	Target	Cq
A01-Ch1	FAM	Hospital 1	*L. pneumophila*	37.1
A01-Ch2	HEX	Hospital 1	legionella spp.	36.2
A01-Ch3	ROX	Hospital 1	leg serogp1	No Ct
A01-Ch4	CY5	Hospital 1	IPC	32.62
A02-Ch1	FAM	Hospital 2	*L. pneumophila*	36.2
A02-Ch2	HEX	Hospital 2	legionella spp.	35.7
A02-Ch3	ROX	Hospital 2	leg serogp1	No Ct
A02-Ch4	CY5	Hospital 2	IPC	32.04
A03-Ch1	FAM	University	*L. pneumophila*	38.38
A03-Ch2	HEX	University	legionella spp.	36.13
A03-Ch3	ROX	University	leg serogp1	No Ct
A03-Ch4	CY5	University	IPC	32.33
A04-Ch1	FAM	Residential	*L. pneumophila*	39.58
A04-Ch2	HEX	Residential	legionella spp.	37.4
A04-Ch3	ROX	Residential	leg serogp1	No Ct
A04-Ch4	CY5	Residential	IPC	32.22

** (IPC) Internal positive control and (Ct) Threshold cycle

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3.4. Antibiotic Profile Study on *L. pneumophila*

The results for antibiotic susceptibility were done for *L. pneumophila* isolates to the following 11 tested antibiotics provided in Table (5) and shown in Figure (7). The range of the zone of inhibition among all antibiotics was (12-61) mm as shown in Figure (6).

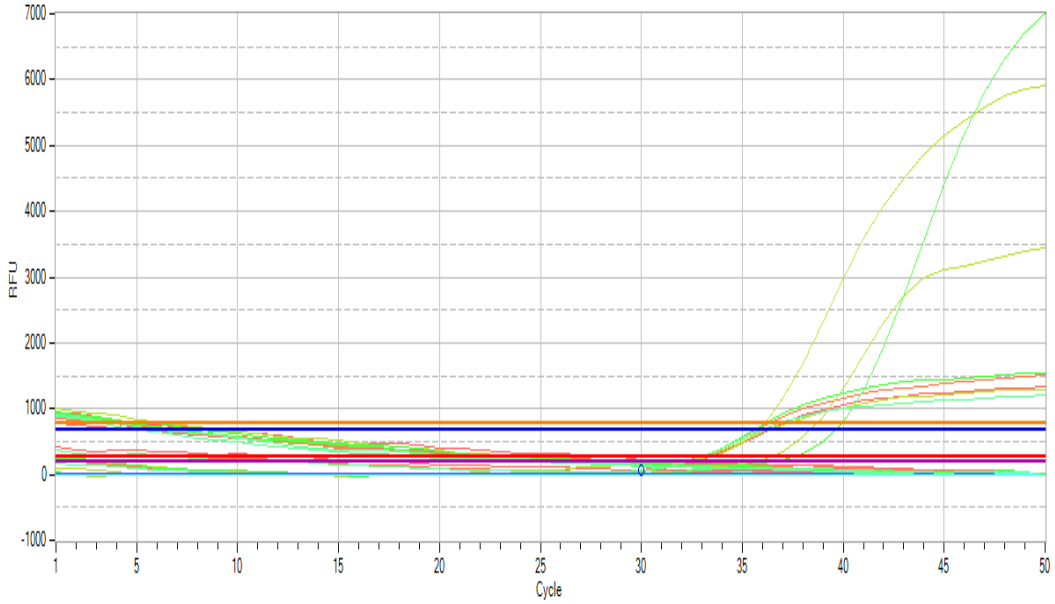

Figure 3. Amplification curve of real-time PCR for *L. pneumophila* isolates.

Table 4. Highest homology isolates based on Identity % values and Query cover.

L. pneumophila **isolates**	**Isolates Accession numbers**	**Accession Numbers**	**Highest homology**	**Identity %**	**Query cover**
17	PP417721	MH412934.1	***L. pneumophila*** isolates AIRBIOTA-BSP3 16S ribosomal RNA gene, partial sequence	100%	94%
18	PP417722	JN983398.1	***L. pneumophila*** strain GC-A13 16S ribosomal RNA gene, partial sequence	93.71%	92%

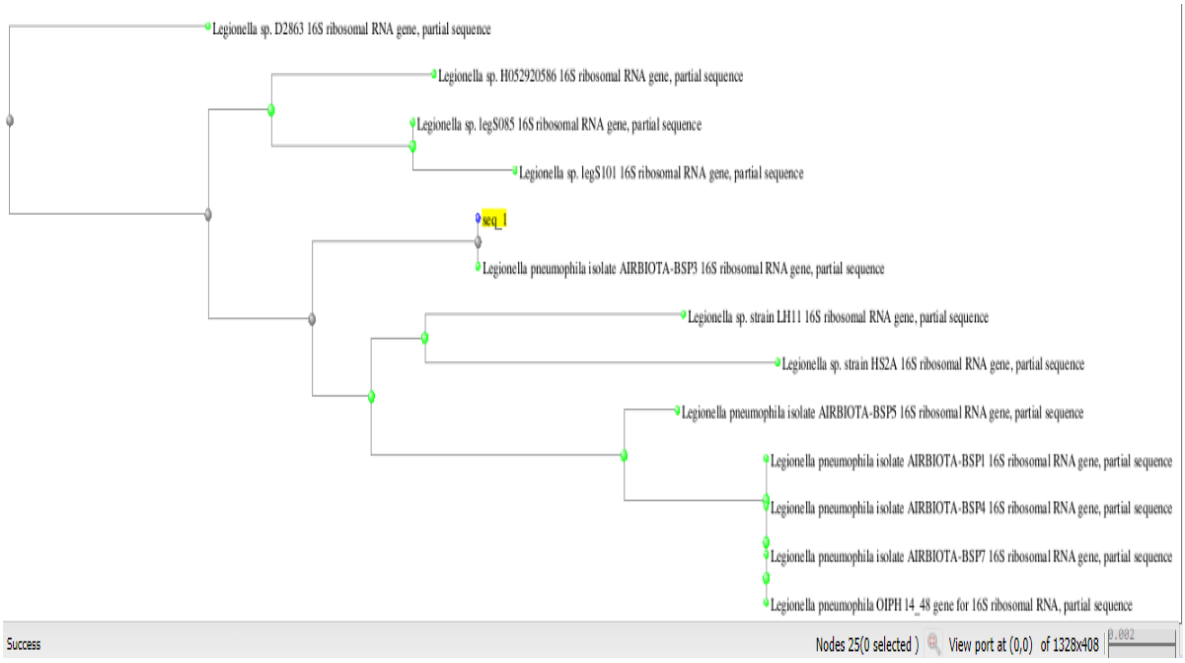

Figure 4. Phylogenetic tree for isolate 17 according to their sequences.

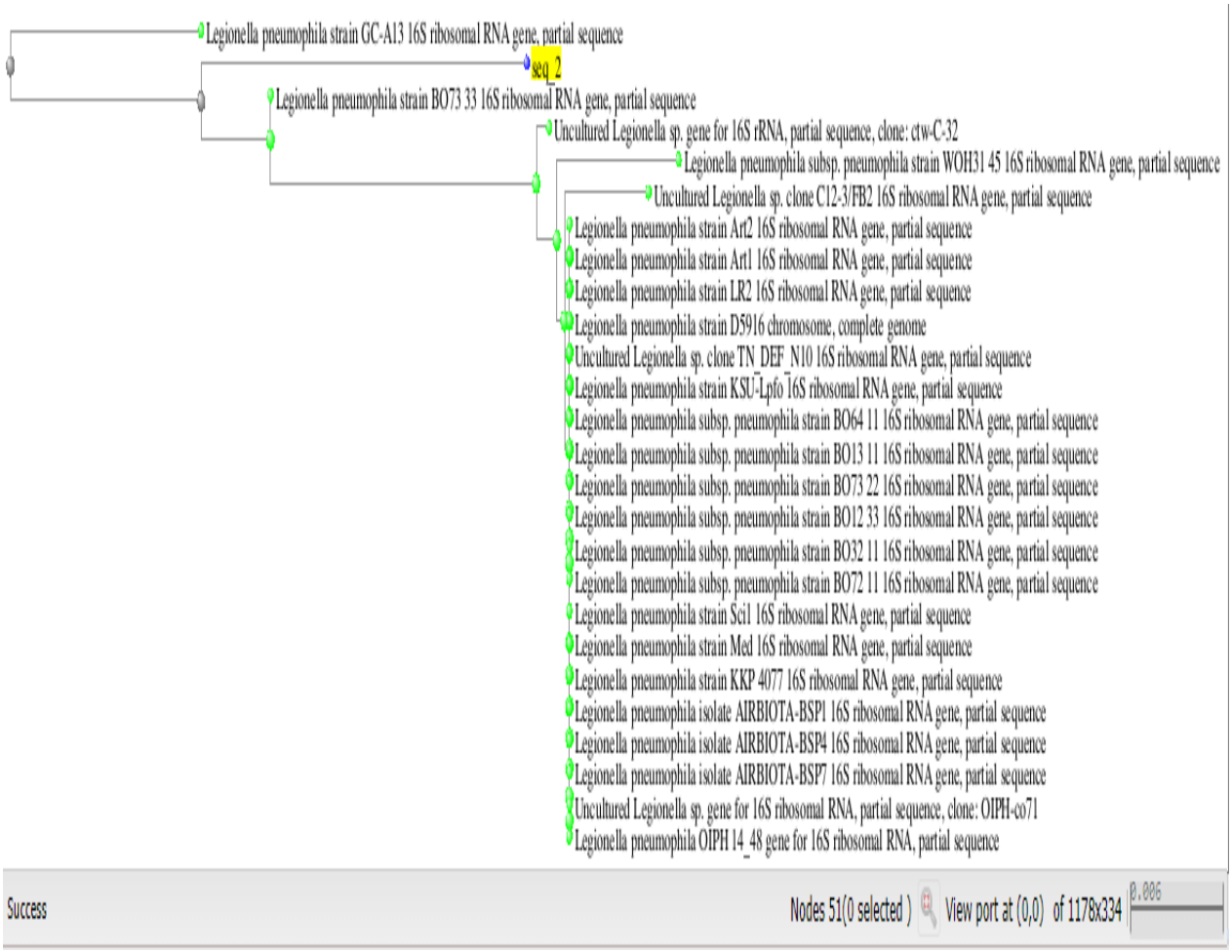

Figure 5. Phylogenetic tree for isolate 18 according to their sequences.

Figure 6. Antimicrobial susceptibility of L. pneumophila to Vancomycin, Rifampin and Clindamycin on BCYE media.

Table 5. Antimicrobial susceptibility pattern of *L. pneumophila* isolates against the tested antibiotics.

L. pneumophila	Zones of Inhibition (mm)										
	Vancomycin	Rifampin	Clarithro-mycin	Meropenem	Imipenem	Erythromycin	Clindamycin	Tetracycline	Levofloxacin	Doxycycline	Ciprofloxacin
Isolate 17	37.7 ± 0.6	34.0 ± 0.0	59.7 ± 0.6	60.3 ± 0.6	56.0 ± 1.0	50.7 ± 0.6	30.3 ± 0.6	40.3 ± 0.6	60.3 ± 0.6	40.3 ± 0.6	60.0 ± 0.0
Isolate 18	15.7 ± 1.2	30.0 ± 0.0	45.0 ± 1.0	53.0 ± 0.0	45.0 ± 0.0	34.7 ± 0.6	17.0 ± 0.0	37.0 ± 0.0	51.0 ± 1.0	27.7 ± 0.6	44.7 ± 0.6
Isolate 19	20.7 ± 1.2	28.3 ± 0.6	46.0 ± 0.0	50.3 ± 0.6	54.0 ± 1.0	40.3 ± 0.6	16.0 ± 1.0	40.3 ± 0.6	60.3 ± 0.6	31.0 ± 1.0	61.0 ± 1.0
Isolate 20	20.3 ± 0.6	25.0 ± 0.0	39.7 ± 0.6	15.7 ± 0.6	27.0 ± 0.0	25.0 ± 0.0	35.3 ± 0.6	12.0 ± 0.0	35.0 ± 0.0	15.3 ± 0.6	35.0 ± 0.0

The findings are presented as the average value ± standard deviation of a minimum of three repeated experiments.

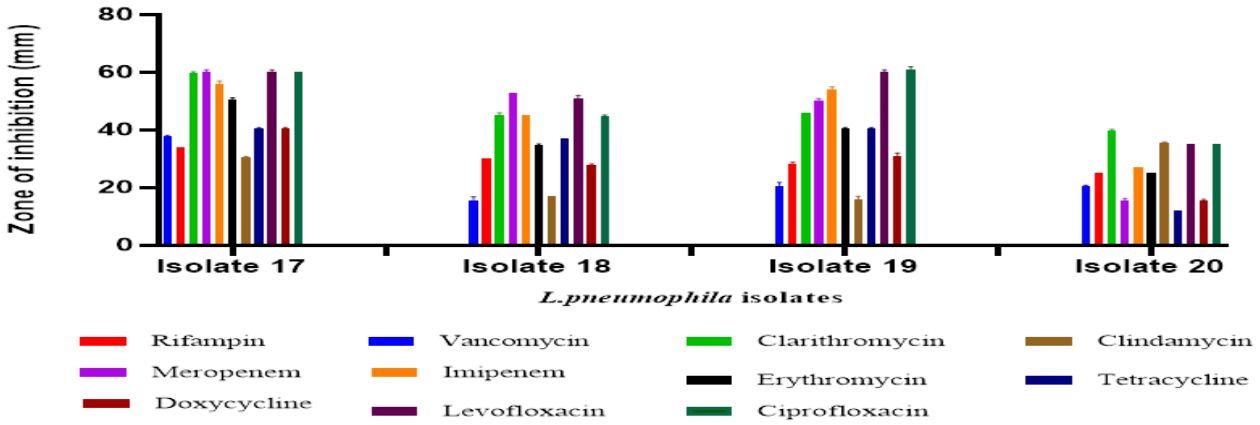

Figure 7. A histogram showed the antimicrobial susceptibility pattern of L. pneumophila isolates using one-way ANOVA. The findings are presented as the average value ± standard deviation of a minimum of three repeated experiments and there was no significant difference between their means of inhibition zones.

4. DISCUSSION

In Egypt, insufficient attention has been directed toward the incidence of *L. pneumophila* in air-conditioning water systems. The contamination of air conditioning systems with Legionella species presents a danger of transmission of Pontiac fever and Legionnaires' disease. Legionnaires' disease (LD) is an atypical type of pneumonia, characterized by a lack of distinct clinical features that can readily differentiate it from other pneumonia types; thus, laboratory investigations are essential for accurate diagnosis [20]. The initial identification of Legionella indicated that inhalation of aerosols from air-handling coolers was responsible for numerous clinical cases [13].

Regular monitoring of air conditioner filters and water systems is crucial for routine pathogen screening to prevent outbreaks of *L. pneumophila*, especially among immunocompromised individuals. The correlation between the risk to human health and the presence of *L. pneumophila* in water samples remains a topic of ongoing discussion. This study successfully utilized a robust methodology involving BCYE agar to isolate Legionella from various air conditioner samples, attaining favorable isolation rates. However, this procedure is not without its drawbacks, including being time-consuming, the possibility of Legionella existing in a viable but non-culturable state, and frequent incidences of false positives and false negatives [21].

Concentrating the water samples proved essential for achieving promising isolation rates, as corroborated by previous studies [13], which employed similar procedures on water samples sourced from lakes, ponds, water tanks, and rivers.

To overcome the restrictions associated with culture-based procedures, molecular techniques, such as quantitative real-time PCR (qPCR), could be employed. qPCR allows for the detection of both viable and non-viable organisms, providing quick, sensitive, and specific identification of Legionella bacteria in environmental water samples [22]. A prior study conducted in Egypt tested 25 water samples, all of which were positive for the Legionella genus [23]. In contrast, our study indicated a culture-based detection of only 4 out of 600 air conditioner filters and water samples (0.7%) yielding Legionella spp.

Legionella is an omnipresent and potentially pathogenic microorganism. The commonly employed antimicrobial agents for treating Legionella pneumonia include macrolides and fluoroquinolones, which are effective against intracellular Legionella spp. capable of surviving and proliferating within human macrophages [24]. Reports concerning the antibiotic susceptibility of Legionella environmental isolates remain limited. Although several studies have shown treatment failures in patients with Legionnaires' disease, the antimicrobial resistance of clinical and environmental isolates has not been extensively recorded. Moreover, regular Legionella spp. susceptibility testing is generally not recommended due to challenges in determining standardized minimal inhibitory concentration (MIC) values [25].

5. CONCLUSION

L. pneumophila infections, which pose significant health risks, can potentially be transmitted through air conditioning systems. Therefore, continuous monitoring of these systems is essential to prevent the proliferation of this bacterium and to mitigate associated health threats.

Supplementary Materials:

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Conflicts of Interest: The authors declare no conflict of interest is associated with this publication

Author Contribution: Allam, E.H. performed the isolation, identification, antibiotic profile study of the bacteria and wrote the paper. Alshareef, W.A. revised and finalized the paper. Elsenduony, M. Shared in the isolation and identification of the bacteria by Real time pcr. Omran, M.E. revised and finalized the paper.

List of Abbreviations:

BCYE: Buffered charcoal yeast extract, Ct: Threshold cycle, DNA: *Deoxy* ribonucleic acid, GVPC: glycine, vancomycin, polymyxin B, and cycloheximide, IPC: Internal positive control, *L. pneumophila*: Legionella pneumophila, LD: Legionnaires' disease, MIC: minimal inhibitory concentration, PCR: Polymerase chain reaction, rRNA: Ribosomal ribonucleic acid, UAT: urine antigen test, ZOI: Zone of inhibition

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