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ASSESSMENT OF THE ANTIBIOTIC SUSCEPTIBILITY AND MINIMUM INHIBITION CONCENTRATION OF *LEGIONELLA PNEUMOPHILA* ISOLATED FROM DIFFERENT SOURCES IN BABYLON PROVINCE

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Abstract

Legionella pneumophila is one of the main pathogenic agents responsible for pneumonia and respiratory tract infections (RTIs). It has high levels of resistance against commonly used antibiotics. The present study was carried out to investigate the antibiotic susceptibility and minimum inhibition concentration of *Legionella* isolated from patients suffered from RTIs and different environmental sources. Totally, 200 respiratory samples and 220 environmental were cultured on buffered charcoal yeast extract agar culture medium (selective for *Legionella*) after treated with (1:10) in a KCl–HCl solution (pH 2.0). Thirty seven (18.5%) out of 200 respiratory samples (sputum and dental wash) were positive for *Legionella pneumophila* and twenty eight (12.7%) out of 220environmental (hospital and domestic water system, air conditioner, showers and tap water), were positive for *Legionella.* Bacterial strains for clinical isolates harbored the highest levels of susceptible with Rifampicin (86.4%) followed by Doxycycline (83.7%), Levofloxacin and Tigecycline (59.4%). Azithromycin and Ciprofloxacin were the least active antibiotics (27.1%) and (29.7%).The other antibiotics exhibited intermediate susceptibility. While the environmental isolates exhibited100% susceptibility to Ciprofloxacin and Rifampicin antibiotic, followed by Doxycycline (89.2%) and Levofloxacin (85.7%). Primary identification of *L. pneumophila* positive strains and their regular treatment with Rifampicin, Doxycycline can reduce the risk of infection and transmission of bacteria.

***Keywords*:** *Legionella pneumophila*; E-test, MIC, Antibiotic susceptibility, Macrolides, Flouroquinolones.

Introduction

Legionellosis is an infectious disease caused by the gram-negative bacilli belonging to the Legionellaceae family. These bacteria are found in aquatic habitats, where they grow in multi species natural biofilms and replicates intracellularly in protozoa, mainly amoeba (Tronel and Hartemann, 2009) Among the *Legionella* genus, the *Legionella pneumophila* is the aetiological agent causing approximately 90% of reported legionellosis cases (Eisenreich and Heuner, 2016; Sepinozen *et al.*, 2017). Healthcare facilities, including hospitals, health centers, residential care dental settings and dialysis units, represent an at risk environment for Legionnaires' disease (LD) transmission because of the frequently old plumbing systems and the use of medical devices from immune compromised patients (Montagna *et al.*, 2017). *Legionella* infection mainly causes two distinct illnesses: Pontiac fever, an acute febrile and self-limiting illness that doesn't require any treatment and the LD, an important cause of community-acquired and hospital–acquired atypical pneumonia, potentially fatal (Hashmi *et al.*, 2016). Respiratory tract infections and pneumonia caused by *L. pneumophila* are usually known by confusion, fever, headache, diarrhea, abdominal pain, chills, non-productive cough and myalgia (Chaudhry *et al.*, 2014). Pneumonia caused by this bacterium often required antibiotic therapy; However, antibiotic resistant strains of this bacterium cause more sever and dangerous diseases for longer periods of time than susceptible strains (De Giglio *et al.*, 2015). According to the recent epidemiological studies, *L. pneumophila* strains show a high prevalence of resistance (50-100%) against commonly used antibiotics including Tigecycline, Ceftriaxone, Rifampicin, Azithromycin, Erythromycin, Moxifloxacin, Ciprofloxacin, Levofloxacin, Doxycycline and Clarythromycin (Harrison *et al.*, 2013).

According to the uncertain role of *Legionella pneumophila* as a causative agent of respiratory tract infections caused us to do this investigation with respect to study the distribution of the bacteria in the respiratory samples taken from patients suffered from respiratory infections as well as study the assessment of the antibiotic susceptibility of the isolates to the currently use antibiotics (Adday *et al.*, 2019).

Materials and Methods

A total of (440) samples were collected from different sources, divided as 200 clinical samples collected from patients (50 dental wash, 130RTIs secretion) and 20 healthy subject. At the time of sampling, information about the age, sex and clinical symptoms of the patients were recorded On the other hand 220 environmental samples (60 domestic water system30 air conditioner samples, 80 tap water samples and 50 showers). Each sample was collected in a sterile glass–stoppered bottle. Isolation of *L. pneumophila* from different sources was performed by culture according to the recommendations of the (Fields *et al.*, 2002). All samples were treated in a KCl–HCl solution to avoid the growth of other un desired bacteria. Concentrations of the treated water samples were carried out by centrifugation at4000 rpm /min supernatant solution was discarded and the sediment was aseptically transferred and cultured on buffered charcoal yeast extract (BCYE CMO 655). The culture media were incubated at 37°C for 36 hours without CO_2 and 5 days in 2.5% CO_2. Selectivity of the medium was subsequently improved by the incorporation of vancomycin and glycine, this selective medium should facilitate the recovery of Legionellaceae. It should be noted that vancomycin and glycine are added to the medium after autoclave and when the temperature was around 50 ºC. Antibiotic susceptibility of the *Legionella* against 10 commonly used antibiotics was determined using the instruction of Clinical and Laboratory Standards Institute guidelines (CLSI, 2019). Susceptibility of isolates were tested against antimicrobial agents (Oxoid, UK). Ceftriaxone (30µg/disk), Azithromycin (15 µg/disk), Erythromycin (15 µg/disk), Ciprofloxacin (5 µg/disk), Doxycycline (30 µg/disk), Rifampicin (5 µg/disk), Tigecycline (15 µg/disk), Moxifloxacin (5 µg/disk), Clarythromycin (2 µg/disk) and Levofloxacin (1 µg/disk).

The minimum bactericidal concentration (MBC)

It is the minimum concentration of an antimicrobial drug that is bactericidal. It is determined by culturing (sub-culturing) broth dilutions that inhibit growth of a bacterial organism. The broth (brain heart infusion broth) dilutions are streaked onto BCYE agar and incubated for72 hours. The MBC is the lowest broth dilution of anti-microbial that prevents growth of the organism on the agar plate. Failure of the organism to grow on the plate implies that only nonviable organisms are present. The use of MBCs has been advocated by some for treatment of serious infections or for treatment of immunosuppressed patients (Wolfson and Hooper, 1985).

E-test analysis

Each isolate was subcultured on BCYE with L-cysteine agar and incubated at 37°C for 5 days. Briefly, the inoculum was prepared by swabbing a portion of growth from the plate using a sterile cotton swab. The swab was transferred to a tube containing 5 ml of sterile water and the turbidity was adjusted to a 0.5 McFarland standard by visual examination. The inoculum was spread on BCYE agar plates and E-test strips were applied to the surface .The plates were incubated at 37°C for 72 h at least before reading the MIC values. The MIC value was determined as the lowest concentration of antibiotic that completely inhibited visible colonies (Bruin *et al.*, 2012). Isolates that had shown the highest MIC values, or revealed no growth during E-test analysis were retested (Erdogan *et al.*, 2010). As there are no official breakpoints for *Legionella spp*. yet, we used the National Committee for Clinical Laboratory Standards (NCCLS) guidelines, as it was described before (Erdogan *et al.*, 2010).

Results

Table (1) represents the total prevalence of *L. pneumophila* in the samples taken from patients suffered from respiratory tract infections. We found that 37 out of 200 samples (18. 5 %) were positive for clinical *Legionella* and 28 (12.7%) from environmental samples.

Table 1: Total prevalence of *Legionella pneumophila* in the respiratory and environmental samples

Source of samples							
Clinical samples	**No.**	**No.(%) of positive growth on BCYE**	**No.(%)f negative growth on BCYE**	**Environmental samples**	**No.**	**No.(%) of positive growth on BCYE**	**No.(%) of negative growth on BCYE**
Sputum	130	29(22.3%)	101(77.7%)	Domestic water system	60	14 (23.3%)	46(76.6%)
Dental wash	50	8 (16 %)	42(84%)	Air conditioner	30	5 (16.6 %)	25(83.3%)
				Showers	50	3 (6%)	47(94%)
Healthy subject	20		20(100%)	Tap water	80	6 (7. 5%)	74(92.5%)
Total	**200**	**37(18.5%)**	**163(81.5%)**		**220**	**28(12 .7%)**	**192(87.2%)**

Antibiotic susceptible properties of *L. pneumophila* isolated from samples taken from patients suffered from RTIs, dental wash and environmental isolates are shown in table (14). Clinical isolates harbored the highest levels of susceptible with Rifampicin (86.4%) followed by Doxycycline (83.7 %), Levofloxacin and Tigecycline (59.4%). Azithromycin and Ciprofloxacin were the least active antibiotics (27.1%) and (29.7%). The other antibiotics exhibited intermediate susceptibility. While the environmental isolates exhibited 100% susceptibility to Ciprofloxacin and Rifampicin antibiotic, followed by Doxycycline (89.2%) and Levofloxacin (85.7%).

Table 2: Antibiotic susceptibility pattern of *Legionella pneumophila* isolated from the clinical and environmental samples

Antimicrobial		**Clinical isolate (n= 37)**			**Environmental isolate (n = 28)**		
		No	**%**	**Biofilm formation**	**No**	**%**	**Biofilm formation**
Azithromycin	S	10	27.1	7(18.9%)	3	10.8%	1 (4 .5%)
	R	27	72.9	25(67.5%)	25	89.2%	21(95.4 %)
Ceftriaxone	S	15	40.6	12(32.4%)	22	78.5%	16(72.7 %)
	R	22	59.4	20(54.0%)	6	21.4%	6 (27.2%)
Ciprofloxacin	S	11	29.7	8(21.6%)	28	100%	22(100 %)
	R	26	70.2	24(64.8%)	0	0 %	0 %
Clarithromycin	S	17	45.9	14(37.8%)	10	35.7%	4(18.1%)
	R	20	54.1	18(48.6%)	18	64.3%	18(81.8%)
Doxycycline	S	31	83.7	26(70.2%)	25	89.2%	19(86.3%)
	R	6	16.2	6(16.2%)	3	17.8%	3 (13.6%)
Erythromycin	S	15	40.5	11(29.7%)	19	67.8%	13(59.0%)
	R	22	59.4	21(56.7%)	9	32.1%	9(40.9%)
Moxifloxacin	S	19	51.3	14(37.8%)	8	28.5%	5(22.7%)
	R	18	48.6	18(48,6%)	20	71.4%	17 (77.2%)

Rifampicin	S	32	86.4	27(72.9%)	28	100 %	22(100%)
	R	5	13.5	5(13.5%)	0	0%	0%
Tigecycline	S	22	59.4	19(51.3%)	18	64.3%	12(54.5%)
	R	15	40.5	13(35.1%)	10	35.7%	10(45.4%)
Levofloxacin	S	22	59.4	19(51.3%)	24	85.7%	18(81.8%)
	R	15	40.5	13(35.1%)	4	14.2%	4(18.1%)

Ceftriaxone (30 µg/disk), Azithromycin (15µg/disk), Ciprofloxacin (5 µg/disk) Clarythromycin (2µg/disk), Doxycycline (30µg/disk), Erythromycin (15 µg/disk) Moxifloxacin (5 µg/disk), Rifampicin (5 µg/disk), Tigecycline (15 µg/disk), Levofloxacin (1 µg/disk).

Table (3) shows MIC and MBC (respectively, the MICs required to inhibit the growth of organisms), of the 10 antibiotics tested for the totality of the clinical *Legionella* isolates.

Table 3 : MIC (E Test) and MBC of antibiotic susceptible of clinical *Legionella* isolates

Antibiotic	Total = 37 isolates No. of sensitive isolates	MIC mg / L	MBC mg / L
Azithromycin	10	12	96
Ceftriaxone	15	2	8
Ciprofloxacin	11	8	16
Clarithromycin	17	16	96
Doxycycline	31	6	24
Erythromycin	15	0 .75	32
Moxifloxacin	19	6	24
Rifampicin	32	12	48
Tigecycline	22	48	128
Levofloxacin	22	4	8

Discussion

The proportion of clinical positive isolates was higher than that of environmental isolates, as in other studies carried out in the UK, England (clinical : 18.5 %. environmental : 12.7%) (Reimer *et al.*, 2010). The ability of microbes to survive in hospital and domestic water reservoir was described more than 30 years ago, and numerous studies have confirmed hospitals water as a source of nosocomial infection (Makin, 2008). Modes of transmission for waterborne infections include direct contact, investigation of water, indirect contact, inhalation of aerosols dispersed from water sources, and aspiration of contaminated water (Sehulster and Chinn, 2016).

Several studies evaluated the variation in sensitivity to antibiotics of environmental and clinical isolates of *Legionella* spp (Xiong *et al.*, 2016). EUCAST, (2015) reported that Erythromycin and Rifampin prevented death of guinea pigs experimentally infected with *L. pneumophila*. Our present data support these studies; that is, Erythromycin and Rifampin were able not only to inhibit multiplication but also to kill *Legionella*. We found that bacterial strains harbored the highest levels of resistance against Ciprofloxacin, Erythromycin and Azithromycin. These are mainly used for treatment of infections caused by Gram-negative bacteria. The main causes for the high prevalence of resistance against these antibiotics are the irregular, excessive and unauthorized prescription such that showed in our results. Several studies were conducted on the prevalence of antibiotic resistance in *Legionella* strains of environmental and clinical samples (Sandalakis *et al.*, 2014). Sikora *et al.* (2017) reported the low levels of *L. pneumophila* resistance against Rifampicin, Doxycycline and Levofloxacin. In fact, these antibiotic agents were effective for treatment of respiratory infections caused by *Legionella* spp. bacteria on several years. According to their studies, these antibiotic hardly penetrated phagocytic cells, however, Erythromycin was taken into cells by an active metabolic process and Rifampin penetrated by simple solubility partition (Melo *et al.*, 2009). The reasons for the efficacy of Erythromycin and Rifampin against intracellular *L. pneumophila* may therefore be their good penetrability and low MIC values. Our study confirms that *Legionella* isolates are inhibited by low concentrations of macrolides and fluoroquinolones (Melo *et al.*, 2009). Among the macrolides Ceftriaxoneis the most active drug for *Legionella* spp with the action of E test (MIC 2 mg/l , MBC 8 mg /l).The MIC value for Doxycycline is consistent with the results of other studies that investigated the susceptibility of clinical isolates (Sikora *et al.*, 2017). Our results indicated that even though Ciprofloxacin and Erythromycin had common MIC (8 mg /land 0.75 mg/l), 40.5 % (15/37) of isolates were considered as low-level resistant in Erythromycin ,while only29.7 % (11/37) were considered as low-level resistant in Ciprofloxacin. This result indicated that Erythromycin was more active than Ciprofloxacin, against the majority of *Legionella* isolates, in accordance with previous studies (Mallegol *et al.*, 2014). The presence of antibiotic less susceptible isolates in the environment is not impossible. *Legionella* bacteria are ubiquitous in aquatic and man-made environments where they can be exposed to antibiotics from medical or veterinary practice, or even from those physically secreted from other microbial. It is known that intracellular life of *Legionella* bacteria protects them by biofilm formation from various toxic agents, including antibiotics used in clinical treatment (Hanlon, 2010).Nevertheless, the presence of antibiotic less susceptible environmental strains could increase the risk of a failed antibiotic treatment in patients with legionellosis.

Conclusions

In conclusion, we identified a large numbers of *L. pneumophila* in the respiratory samples of patients suffered from respiratory tract infections as well as their antibiotic resistance pattern. We found that judicious and regular prescription of Rifampicin, Doxycycline and Erythromycincan control the risk of respiratory tract infections due to the *L. pneumophila*.

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