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Occurrence of Legionella spp. in Clinical and Environmental Samples in Babylon Province

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Abstract--- *This study was designed to determine the occurrence of Legionella spp. recovered from clinical(sputum and dental wash) and environmental (hospital and domestic water system, air conditioner, showers and tap water)samples using standard biochemical testss. The all samples were diluted (1:10) in a KCl–HCl solution (pH 2.0), this was mixed and incubated at room temperature for 4s min, to avoid the growth of other undesired bacteria and to facilitate the better isolation of Legionella spp. Supernatant was discarded and the sediment was transferred and cultured on buffered buffered yeast extract (BCYE) agar culture medium. The culture media were incubated at 37 °C for36 hours without CO_2 and 5 days in 2.5 % CO_2. Degrees of the occurrence of Legionellapneumophilain clinical remains high (18.5%) in comparison with environmental samples (12.7%).*

Keywords--- *Babylon Province, Occurrence of Legionella, Clinical and Environmental*

I. INTRODUCTION

Legionnaires' disease is a form of atypical pneumonia caused by any type of *Legionella* bacterias (Fields *et al*, 2002). Signs and symptoms include cough, shortness of breath, high fever, muscle pains, headache, nausea, and diarrhea may also occur (Palmer *et al*,2016). This often begins2- 10 days after exposure. The infection caused by the inhalation of *Legionella* depends on the ability of these organisms to enter and to multiply within alveolar macrophages causing the destruction of these phagocytes and damage to the pulmonary tissues (Cunha *et al* 2016).The bacterium is found naturally in fresh water, it can contaminate hot water tanks, hot tubs, and air conditioners (Heijnsbergen *et al*, 2015). It is usually spread by breathing in mist that contains these bacteria. The bacterium can also occur when contaminated water is aspirated (Newton *et al*, 2010).Risk factors for infection include older age, history of smoking, chronic lung disease, and poor immune function(CDC, 2017).The bacterium was identifiedin1976, which caused the epidemic of pneumonia that spread during a convent of the American Legion in Philadelphia, USA (Fraser *et al*, 1977). After its identification, *Legionella pneumophila* was shortly characterized as a ubiquitous bacterium that parasitizes free - living environmental protozoa so this finding paved the way for the concept that the ecology and pathogenes is of *L. pneumophila* are closely linked (Escoll *et al*,2013). *Legionella* do not form spores. There are two major phases to the life cycle, The first is called the replicative phase, during this period, the bacteria are non motile and have a low toxicity, in the second phase, called the infectious phase, the bacteria are shorter and thicker, they have developed flagella and are highly toxic (Khodr *et al*,2016).They have the capability of surviving within biofilms, particularly in man-made water systems, in these types of environments, extracellular growths soften a necessity, and the biofilms permit this growth. Evidence suggests that a majority, if not all, the bacteria are associated with biofilms (Cervero *et al*,2015).The *Legionella*

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bacterium also causes Pontiac fever, a milder illness resembling the flu. Separately or together, the two illnesses are sometimes called legionellosis (Molmeret *et al*, 2004). Pontiac fever usually clears on its own, but untreated legionnaires' disease (LD) can be fatal. Although prompt treatment with antibiotics usually cures legionnaires' disease, some people continue to experience problems after treatment. iPontiac fever doesn't infect lungs, and symptoms usually clear within two to five days (Diederen, 2008). However, the current study was aimed to :

*Determinate the occurrence of *Legionella* spp recovered from clinical and environmental samples using standard biochemical tests.

II. MATERIALS AND METHODS

Sampling, Processing, and Enrichment. During May s– September 2018, two hundred clinical samples (130 sputum, 50 dental wash and 20 control healthy subjects) and two hundred twenty water samples (about 500 ml for each) were collected by aseptically containers (60 hospitals and domestic water system,30 air conditioner, 50 showers and 80 tap water). Isolation of *Legionella* from different sources was performed by culture according to the recommendations of the (Tronel and Hartemann, 2009). All samples were treated ina KCl–HCl solution, to avoid the growth of other un desired bacteria. Concentrations of the treated water samples were carried out by centrifugation at 4000 rpm /min, supernatant solution was discarded and the sediment was aseptically transferred and cultured on buffered charcoal yeast extract (BCYE CMO 655). Manufacturing details, activated charcoal 2.0 g /L, L cysteine 0.04 %,glycine 0.03%, ferric pyrophosphate 0.25 g/ L, vancomycin 5μg/mL, Yeast extract 10.0 g/L. The culture media were incubated at 37°Cfor 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 ∘C. The detection of biofilm formation was done by tube method according to (Franklin *et al*,2015).

III. RESULTS

Two hundred clinical samples were collected from (130 sputum, 50 dental wash and 20 healthy subject). Infection rates of sputum, dental wash with *Legionella* spp, were 22.3 % and 16%. On other hand two hundred and twenty environmental samples collected from (60 domestic and hospital water system,30 air conditioner, 50 showers and 80 tap water. The rates of contamination with *Legionella* spp. were 23.3%, 16.6%, 6% and 7.5 % (table 1).

Table 1: Distribution of *Legionella* spp in clinical 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%)**

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Among the patient population studied, out of the 180clinical isolates were found to be culture positive for *L. pneumophila*, majority were males 26(56.7%)table (2, 3), and at>50 years of age, table (4).

Table 2: Number and percentage of positive isolates on BCYE according to sex obtained from all clinical isolates

No. samples	No, (%) positive culture	Sex
180	26 (56.7 %)	Male
	11 (29.7 %)	Female
Total all positive	37 (18.5%)	

Table 3: Number and percentage of positive isolates on BCYE according to sex obtained from sputum and dental wash

Source	No	Sex	No(%) of positive isolates
Sputum	29	Male	21 (72.4 %)
		Female	8 (27.5 %)
Dental wash	8	Male	5 (62.5%)
		Female	3 (37.5%)
Total	37		

Table 4: Number and percentage of positive isolates on BCYE according to age

Age	No (%) of positive culture on BCYE
20 – 30	5 (13.5 %)
31 – 40	2 (5.4 %)
41 - 50	9 (24.3 %)
>50	21 (56.7%)
Total	37 (99.9%)

Among the risk factors smoking habit was found in 13 (61.9%) patientss, Tobacco smoke exposure increases susceptibility to respiratory tract infections, including pneumonia and Legionnaires disease; table (5).

Table 5: Number (%) positive male isolates on BCYE according to smoking

Total of positive male isolates	Smoking	No.(%) of positive isolates
21	Positive	13 (61.9 %)
	Negative	8(38.1 %)
Total		21 (100%)

Of the 37 clinical isolates tested, 22 strains (59.4%) were observed to form biofilms in vitro by tube method at levels greater than the other and 17 (60.7%) from 28 environmental isolates form strong biofilm formation by same method table (6).

Table 6: Biofilm formation of isolates recovered from clinical and environmental samples

Source of isolates	No. of positive culture	Biofilm formation		
		Strong	Weak	Non
Clinical	37	22(59.4%)	10(27.0%)	5(13.5%)
Environmental	28	17(60.7%)	5(17.8%)	6(21.4%)
Total	65	39	15	11

The physiological characteristics of the 37 Clinical isolates and 28 environmental isolates of *L. pneumophila*were slender, Gram-negative, non-acid-fast rods having the morphological and staining properties of

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this species. They did not readily take up safranin in the gram-stain procedure. All 37 clinical isolates liquefied gelatin and were catalase positive. Oxidase test was negative for16isolates and positive for21 isolates. Some environmental isolates of *L. pneumophila*were oxidase negative when tested under the same conditions table (7).

Table 7: Biochemical characters of *Legionella spp*

Biochemical test	Clinical isolates N= 37	Environmental N= 28
Heamolysis activity	_	_
Oxidase test	±	±
Catalase test	+	+
Urease test	_	_
Motility test	+	+
Simmon citrate	_	_
Gelatinase test	+	±

± Variation, _ Negative, + positive

IV. DISCUSSION

Due to the lack of characteristic symptoms of the disease, diagnosis of Legionnaires' disease may be difficult. It is therefore vital to have access to accurate diagnostic methods in order to confirm clinical suspicion. The most commonly used method today for the diagnosis of LD is respiratory sample culture (Mercante, 2015).

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 % vs. environmental : 12.7 %) (Reimer *et al*,2010), suggested that the some epitope structures is easily lost during adaptation to environments when there is no pressure to retain human pathogenicity (Kozak, 2014).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, ingestion of water, indirect contact, inhalation of aerosols dispersed from water sources, and aspiration of contaminated water (Sehulster and Chinn, 2016). Several factors could be attributed to the contamination of water such as the age of the distribution system, the quality of the delivered water, defective chlorination (Phin *et al*., 2014).

The bacterial contamination occurs because of regrowth of microorganisms in biofilms which are formed on interior surfaces of water pipes. Biological activity in biofilms is controlled by nutrient content of water, temperature, and residual chlorine (Bonadonna L *et al*.2009).In the present study, the degree of contamination hospital and domestic water system with *Legionella* was higher (23.3%) than tap water (7.5%).The colonization of *Legionella* bacteria in the domestic water system poses a significant problem because it is the water source for the occupant's use. In addition, *Legionella* bacteria are difficult to control in the domestic water system because they may hide and survive in the biofilm on the surface of the pipes (Beloin *et al*,2014).It also can be isolated even in the presence of 2.5 -- 3ppmresidualfree chlorine (Loret and Greub,2010). A number of laboratory-based studies have demonstrated the colonization of heterogeneous biofilm developed from tap water microorganisms by pathogens such as *L.pneumophila* (Hilbi *et al*.,2011).We showed that (60.7 %) of these bacteria are important opportunistic

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pathogens which can be involved in biofilms associated contamination of domestic systems, this results was compatible with (Richards *et al*, 2015).This bacterium can also survive at lower temperatures in drinking water environments (Jemba *et al*, 2015).*L. pneumophila*is known as cause of both community acquired and hospital associated pneumonia (Cunha, 2016). Several reports have demonstrated that the major sources for Legionnaires'diseasearethepotablewatersystemsoflargebuildingsincludinghospitals, nursing home, and hotels (Osawa and Shigemura, 2014).We attempted to demonstrate the hemolytic activity of the environmental and clinical isolates of *L. Pneumophila* on blood agar, as described by (Azad *et al*,2011). Both surface and subsurface hemolysis was absent so that it was not possible to characterize the hemolytic reactions because *Legionella* bacteria produce proteases. These enzymes may play roles in hemolysis and are considered weak virulence factors (Dowling *et al*, 1992). Active smokers and those exposed to secondhand smoke are at increased risk of bacterial infection. Tobacco smoke exposure increases susceptibility to respiratory tract infections, including pneumonia and Legionnaires disease. In current study (61.9 %) of isolates were to male smokers, these result was compatible with (Murphy, 2006).

V. CONCLUSION

More *Legionella* species have been identified to be associated with disease. the emergence of the *L. pneumophila* as a major respiratory pathogen in 37 clinical isolates. Our experience highlights the importance of considering the possibility of infections in patients with pneumonia who were negative results with Genix device that was specialize for Tuberculosis diagnosis. Patients who are smokers, or who are immunosuppressed should be treated with selective laboratory culture by buffered charcoal yeast extract media.

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