Prevalence of Legionella spp. and Helicobacter pylori in different water resources in Egypt

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Abstract

The main objective of this investigation was to detect *Legionella* spp. and *Helicobacter pylori* in different types of water resources. One hundred seventy five different water samples were collected from Egypt. Water samples were collected from Nile River (Rossita Branch), the Mediterranean Sea at Marsa Matroh shore, El-Rahawy Drain, hospital wastewater and groundwater. *Legionella* spp. and *Helicobacter pylori* were determined using membrane filtration and spread plate techniques. *Legionella* spp. and *H. pylori* were detected using culture methods on selective media in 25 and 33% of the total examined water samples, respectively. The biochemical tests for *Legionella* spp. showed that, 6 (35%) out of 17 might belong to *L. pneumophila* and 4 (23%) out of 17 belonged to non-pneumophila *Legionella* species. Eight out 27 (29%) isolates showed positive results for urease, catalase, oxidase, motility tests and negative nitrate reduction were confirmed as *H. pylori.* The results concluded that further monitoring and identification should be carried out in the future for the presence pathogens in water resources.

Keywords

Legionella spp., H. pylori, Nile River, El-Rahawy Drain, Ground water

Introduction

Water plays a vital role for the sound health of each person and is essential for plant life. The human body comprises approximately 70 % of water (Pant, 2004). Waterborne diseases are a major worldwide threat to public health, despite significant advances in water and wastewater treatment technology. Prüss *et al.* (2002) estimated that, waterborne disease is responsible for 4.0% of all deaths and 5.7% of the total disease burden worldwide. It is estimated that about 3.0%

of all deaths worldwide are attributed to unsafe water caused by poor sanitation and hygiene, a problem particularly acute in the developing countries (WHO, 2009).

Microbial pollutants of gastroenteritis including *Campylobacter, Salmonella, Shigella, Legionella, Aeromonas* and emerging pathogens such as *Helicobacter pylori* are contaminating water (Sidhu and Toze, 2009). It is well known that *Legionella* spp. causes Pontiac fever and legionnaires disease (Sabria, 2004). Water is the major reservoir for *Legionella* spp. and *Legionella* spp. have been detected in as many as 40- 80% of freshwater environments (Lau and Ashbolt, 2009).

Helicobacter pylori causes up to 95% of duodenal ulcers and 80% of stomach ulcers and between 50 and 90% of all stomach cancers (Rupnow *et al.*, 2000; Testerman and Morris, 2014). In developing countries, 70 to 90% of the population carries *H. pylori* (Dunn *et al.*, 1997; Obiageli and Ivan, 2016). *H. pylori* is considered as waterborne pathogens transmitted in different water environment including drinking water, wastewater and sea water (Queralt *et al.*, 2005).

Cultural method remains to be the golden standard for the identification of *Legionella* spp. Moreover, Center of Disease Control and Prevention (CDC) suggests routine environmental culturing of water supply for *Legionella* spp. (Sabria, 2004). There is no standard method for detecting *H. pylori* in environmental samples. The accuracy of results varies according to the sensitivities and specificities of the detection methods employed (Lu *et al.*, 2002). Monitoring of *Legionella* spp. and *H. pylori* in different water sources have been recommended by many research works and agents (Yang, 2004; Samendra et al., 2014). Thus, this investigation was carried out to detect and enumerate *Legionella* spp. and *H. pylori* in different water sources from Egypt.

Material and Methods

Sampling sites

Groundwater samples

Forty untreated groundwater samples were collected from twenty wells (2 runs) from El-Kharga, El-Dakhla and Paris Oases, New Valley Governorate. In addition to, forty treated groundwater samples were collected from Qalyubia Governorate from groundwater treatment plants as follow; El- Naseria, Tokh and Qalyub.

Nile River water samples

Fifty water samples were collected from (10 sites) of Nile River (Rossita Branch) at Giza Governorate during five months. Sampling sites from south to north were as follow; 500 m, 400 m, 300 m before the mixing point with El-Rahawy Drain, the mixing point (the point at which El-

Rahawy Drain water is directly discharged and mixed with Nile River water (Rossita Branch)), 100 m, 200 m, 300 m, 400 m, 500 m and 600 m after the mixing point**.**

El-Rahawy Drain water samples

Twenty samples were collected along El-Rahawy Drain during five months, (four samples per month) from four sampling sites; beginning of El-Rahawy village, after 1 Km, after 2 Km and after 3 Km.

Sea water samples

Fifteen coastal water samples were collected from the Mediterranean Sea at Marsa Matroh Governorate from five public beaches. Coastal samples were collected 30 m distance from the shore of the sea.

Hospital wastewater samples

Ten separate wastewater samples were collected from El-Kasr El-Aini hospitals before mixing with sewerage system.

Sampling procedure

Samples were collected in a wide mouth using sterile sampling glass bottles and preserved in icebox and transferred to the laboratory within 2- 6 h.

Determination of total viable bacterial counts (TVBC)

The TVBC at both 37 and 22^{o}C were determined to outstand on the general microbial profile of microbial load. Determination of TVBC was carried out according to APHA (2012) by using poured plate technique.

Identification of *Legionella* spp. isolates

Detection of *Legionella* spp. and *H. pylori* were carried out using MF technique plated on buffered charcoal yeast extract agar (BCYE) media and Columbia agar media, respectively. Biochemical identification of *Legionella* spp. was carried out according to Brenner *et al.* (1984) and APHA (2012). Suspected colony which exhibited growth on BCYE, and no growth on both BCYE without L-cysteine and nutrient broth were presumed to be *Legionella* spp. The isolates showed negative results for urease and nitrate reduction, and positive results for motility, catalase, oxidase, gelatin hydrolysis tests and exhibited blue white fluorescence under UV at 365 nm were confirmed as *Legionella pneumophila*. While non-motile isolates, which showed negative for

urease and nitrate reduction, oxidase and non-gelatin hydrolysis and exhibited blue white fluorescence under UV at 365 nm were confirmed as non-pneumophila *Legionella* species.

Identification of *H. pylori* isolates

Biochemical identification of *H. pylori* was carried out according to Stephen *et al.* (1986). Suspected *H. pylori* isolated from Columbia agar supplemented with 5% sheep blood and persevered on BHI slant (10% glycerol). The isolates which showed urease, catalase, oxidase, motility tests positive and nitrate reduction negative were confirmed as *H. pylori.*

Statistical analysis

The relationship between TVBC and *Legionella* spp. and *H. pylori* were carried out using linear correlation (Person correlation) ($P \leq 0.005$ and $P \leq 0.05$). The statistical analyses were performed on the counts of 50 Nile River water samples, 20 El-Rahawy Drain water samples and 10 hospital wastewater samples out of 175 all collected water samples**.**

Results

Association of TVBC with *Legionella* spp. and *H. pylori* in water samples

Table (1) shows the range and average counts of TVBC at 37 and 22^{o}C, *Legionella* spp. and *H. pylori*. The counts varied according to water source, the highest counts were found in El-Rahawy Drain water samples for *Legionella* spp. and *H. pylori* followed by Nile River water samples, then hospital wastewater.

In Nile River water samples, TVBC at 37 and 22^{o}C ranged between 30 and $8.4x10^5$ CFU/ml and 7x10 and $9.6x10^5$ CFU/ml, respectively. On the other hand, *Legionella* spp. and *H. pylori* fluctuated from 1.1x10 to $9.3x10^3$ and 1.6 x10 to $5.7x10^3$ CFU/100ml, respectively (Table 1).

El-Rahawy Drain water samples showed average counts of TVBC at 37 and 22^{o}C $8.5x10^5$ and $1.6x10^6$ CFU/ml, respectively. TVBC at 37^{o}C ranged between $1.1x10^4$ and $4.4x10^6$ CFU/ml, while at 22^{o}C the counts ranged between $1.2x10^4$ and $8.0x10^6$ CFU/ml, whereas, *Legionella* spp. and *H. pylori* were $2.5x10^3$ and $3.1x10^3$ CFU/100ml, respectively (Table 1).

In hospital wastewater samples, the average counts of TVBC at 37 and 22^{o}C were $6.4x10^4$ and $5.9x10^4$ CFU/ml, respectively. TVBC at 37^{o}C ranged between $4.0x10^3$ and $2.0x10^5$ CFU/ml and at 22^{o}C ranged between $1.6x10^3$- $3.0x10^5$ CFU/ml. On the other hand, the average counts of *Legionella* spp. and *H. pylori* were $2.1x10^3$ and $2.2x10^2$ CFU/100ml, respectively (Table 1).

The results of treated and untreated groundwater, in addition to, sea water samples showed absence of the above mentioned organisms (Tables 1 and 2).

Legionella spp. and *H. pylori* were detected in 44 and 59 out of 175 examined water samples (25% and 33%) using culture methods, respectively (Table 2). In the fifty Nile River water samples; the prevalence *Legionella* spp. and *H. pylori* were 18 (36%) and 33 (66%) respectively. In case of 20 El-Rahawy Drain water samples, the prevalence of *H. pylori* was 20 out of 20 (100%), while the prevalence of *Legionella* spp. was 16 out of 20 (80%) (Table 2).

In 10 hospital wastewater samples, *Legionella* spp. and *H. pylori* was present in 10 (100%), 6 (60%) out of 10 hospital wastewater samples, respectively (Tables 1 and 2). Generally, the occurrences of *Legionella* spp. and *H. pylori* in all collected water samples were 44 (25%) and 59 (33%) out of 175 water samples using BCYE agar and Columbia blood agar, respectively (Table 2).

Table 1: Range and average values of TVBC, *Legionella* spp. and *H. pylori* from different water samples

Water source	No. of samples		TVBC (CFU/ml)		CFU/100ml	
			37°C	22°C	*Legionella* spp.	*H. pylori*
Nile River	50	Range	3.0x10-$8.4x10^5$	7.0x10-$9.6x10^5$	1.1 x10-$9.3x10^3$	1.6 x10-$5.7x10^3$
		Average	$1.7x10^5$	$1.4x10^5$	$3.3x10^2$	$2.7x10^2$
El-Rahway Drain	20	Range	$1.1x10^4$-$4.4x10^6$	$1.2x10^4$-$8.0x10^6$	2.7x10-$8.3x10^3$	$4.7x10^2$-$9.9x10^3$
		Average	$8.5x10^5$	$1.6x10^6$	$2.5x10^3$	$3.1x10^3$
Hospital wastewater	10	Range	$4.0x10^3$-$2.0x10^5$	$1.6x10^3$-$3.0x10^5$	2.0x102-$9.0x10^3$	$2.1x10^2$-$9.0x10^3$
		Average	$6.4x10^4$	$5.9x10^4$	$2.1x10^3$	$2.2x10^2$
Untreated ground water	40	Range	1.0- $1.1x10^3$	2.0- $1.2x10^3$	ND	ND
		Average	1.52 $x10^2$	1.8 $x10^2$	---	---
Treated ground water	40	Range	1.0- 2.2 x10	2.0- 2.8 x 10^2	ND	ND
		Average	7.0	1.0x10	---	---
Sea water	15	Range	$1.0x10^2$-$5.2x10^3$	$1.0x10^2$-$5.6x10^3$	ND	ND
		Average	$9.2x10^2$	$1.4x10^3$	---	---

ND: Not Detected.

Table 2: Number and percentage of *Legionella* spp. and *H. pylori* by culture methods from different water samples

Water source	No. of samples	Culture methods (CFU/100ml)	
		Legionella spp.	*H. pylori*
Nile River	50	18 (36%)	33 (66%)

Water source	No. of samples	Culture methods (CFU/100ml)	
		Legionella spp.	*H. pylori*
El-Rahawy Drain	20	16 (80%)	20 (100%)
Hospital wastewater	10	10 (100%)	6 (60)
Untreated ground water	40	0 (0%)	0 (0%)
Treated ground water	40	0 (0%)	0 (0%)
Sea water	15	0 (0%)	0 (0%)
Total samples	175	44 (25%)	59 (33%)

Percentage was calculated according to each type of examined water samples

Identification of *Legionella* spp. by biochemical tests

Grayish-white colonies that grew on BCYE agar were picked up and preserved on brain heart infusion broth (BHI) (containing 10% glycerol).

Presumed *Legionella* colonies were simultaneously inoculated on BCYE agar, BCYE without L-cysteine and nutrient broth. Seventeen out of 30 (56.6%) colonies grow on BCYE agar only and showed no growth on both BCYE without L-cysteine and nutrient broth were assumed to be *Legionella* and were subjected to further tests (Table III-22).

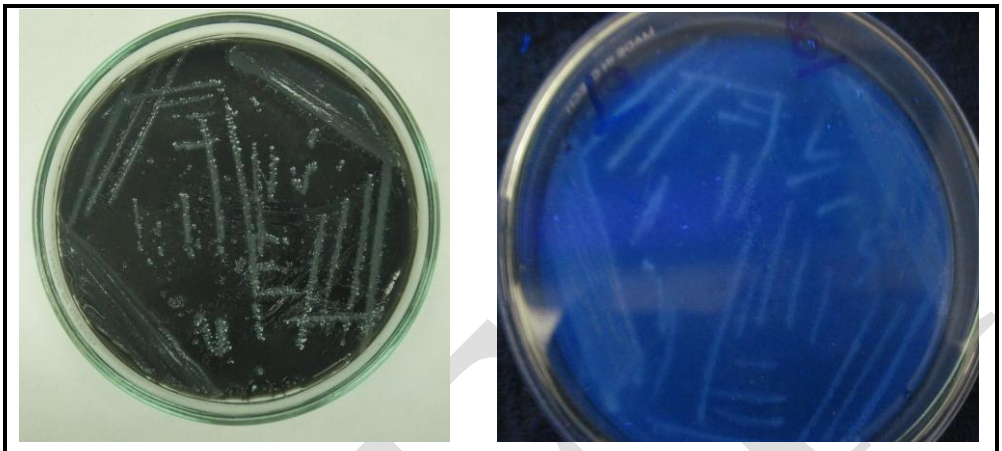
The next step was the exposure of colonies grown on BCYE media to UV lamp 365 nm, colonies which exhibited blue white fluorescence under UV at high wavelength (365 nm), and were at the same time negative for urease and nitrate reduction tests indicated that they are more likely to belong to *Legionella* spp. The biochemical distinguishing tests; gelatin hydrolysis, oxidase and motility tests showed that, 6 (35%) out of 17 might belonged to *L. pneumophila* and 4 (23%) out of 17 belonged to other *Legionella* species (Table 4) and (Figure 1).

Table 4: Biochemical tests of *Legionella* isolates

Total Isolates (30)	**Growth on BCYE With Cysteine**	**Growth on Nutrient broth**	**Growth on BCYE without Cysteine**	**Under UV (365 nm)**	**Gelatin hydrolysis**	**Urease test**	**Nitrate reduction**	**Oxidase**	**Catalase**	**Motility**
Presumptive test Unknown spp. (13)	+	+	+							
L. pneumophila (6)	+	-	-	BW	+	-	-	+	+	+
Other *Legionella*	+	-	-	BW	-	-	-	+/-	+	-

spp. (4)										
Non *Legionella* spp. (7)	+	-	-	-	+/-	+	+	+/-	+	+

BW: Blue White, **+:** Positive, **-:** Negative, **+/-:** Variable



(a) **b)**

Figure 1 (a). Suspected *Legionella spp*. on BCYE agar media (b) Confirmed *Legionella* on BCYE agar media under UV (365 nm)

Identification of *H. pylori* by biochemical tests

Presumptive 27 *H. pylori* isolates were isolated on Columbia agar supplemented with 5% sheep blood and persevered on BHI slant (10% glycerol). Eight out 27 (29%) isolates showed urease, catalase, oxidase, motility tests positive and nitrate reduction negative were confirmed as *H. pylori*. Nineteen out 27 isolates may be other *Helicobacter* spp.

Statistical analysis

Table (3) shows the relationship between TVBC and *Legionella* spp. and *H. pylori* which carried out using linear correlation. In Nile River water samples, there was positive correction with high significant between TVBC at both 37 and 22^{o}C with *Legionella* spp. and not significant correction with *H. pylori*.

Regarding El-Rahawy Drain water samples, there was positive correction with no significant correction observed between TVBC at 37^{o}C with *Legionella* spp. and negative correction between TVBC at 22^{o}C with *Legionella spp*. Moreover, there was negative correction with no significant correction observed between TVBC at both 37 and 22 ^{o}C with *H. pylori*.

In hospital wastewater samples, there was negative correction with no significant correction observed between TVBC at both 37 and 22^{o}C with *Legionella* spp.. While, there was positive

correction with no significant correction observed between TVBC at 37°C with *H. pylori* and highly significant with TVBC at 22°C.

Table 3. Linear correlation between pathogenic bacteria TVBC at 37 and 22°C in Nile River water samples

Water Type	Bacterial indicators and other pathogens	*Legionella* spp.		*H. pylori*	
		r	*p*-Value	r	*p*-Value
Nile River	TVBC at 37°C	0.303	0.032*	0.233	0.103
	TVBC at 22°C	0.285	0.044*	0.099	0.495
El- Rahawy Drain	TVBC at 37°C	0.005	0.984	-0.060	0.800
	TVBC at 22°C	-0.165	0.486	-0.153	0.521
Hospital wastewater	TVBC at 37°C	- 0.320	0.368	0.412	0.237
	TVBC at 22°C	- 0.250	0.485	0.756	0.011*

*Statistically significant (P≤0.05)
** Statistically highly significant (P≤0.005)
r: correlation coefficient (Pearson correlation)

Discussion

In this study, it is important to mention that, *Legionella* spp. and *H. pylori* were detected by culture methods (BCYE agar supplemented with CCVC and Columbia agar for *Legionella* spp and *H. pylori*, respectively), and the suspected colonies were confirmed by biochemical tests, moreover *Legionella* spp. were confirmed by giving blue white fluorescence under UV at 365 nm. Several attempts were carried out to confirm *Legionella* spp. and *H. pylori* using specific primers were unsuccessful, this may be due to low sensitivity of selected primers or failure in optimization the condition.

The results showed that *Legionella* spp. was not detected in groundwater and sea water. While it was detected in 36% of Nile River water samples, 80% of El-Rahawy Drain water samples and 100% of hospital wastewater samples. The averages counts of *Legionella* spp. were $3.3x10^2$, $2.5x10^3$ and $2.1x10^3$ CFU/100ml, respectively. The high counts of *Legionella* spp. in this study revealed that, most waterborne pathogens come from animal and human fecal wastes. *Legionella* spp. occurs naturally in water and can multiply in response to environmental changes. Whereas, *Legionella* spp. protect themselves from environmental stress and survive for long time in different water sources. The survivability of *Legionella* spp. in water was attributed to its ability to infect a total of 13 species of amoebae and two species of ciliated protozoa (Fields, 1996; Watanabe *et al.*, 2016).

Culture method is the golden standard to detect *Legionella* spp. in clinical and environmental samples. The sensitivity of culture was approximately 70% (OSHA, 2001). There are many reasons of low sensitivity of culture methods (1) *Legionella* spp. become VBNC under the stress

condition in water samples (2) number of *Legionella* spp. might be low, could not be able concentrated in definite volume of water samples. (3) there is no selective enrichment media for *Legionella* spp. to enhance the growth of these inactive forms in environmental samples (Yamamoto, 1996).

Wojcik-Fatla1 *et al.* (2012), examined 35 samples of hot tap water from an urban municipal water supply system and 5 samples of cold well water, they found that 62% of samples were positive for *Legionella* spp. using BCYE agar. In addition to, Jomkumsing (2003) examined one hundred and sixty nine tap water and swab samples collected from the hotels, hospitals, shopping center, College of Public Health; and river and canal surface water in Thailand. He found that, 2.4% of tested water samples were positive for *L. pneumophila* by culture methods. Also, Buchbinder *et al.* (2002) examined one hundred water samples (32 from clinical units and 68 from private households). They found that, 35% water samples were positive by culture methods (22 *L. pneumophila*; 2 *non-pneumophila* species) using BCYE agar. Moreover, Tishyadhigma *et al.* (1995) reported that, *Legionella* spp. was detected in 9.6% of river, 1.4% of canal samples, and 8.7% ponds samples by culture methods.

To date, there is no standard method for detecting *H. pylori* in environmental samples. The results of the present study show, *H. pylori* was not detected in groundwater, sea water. It was detected in 66% of Nile River water samples, 100% of El-Rahawy Drain water samples and 60% of hospital wastewater samples. The averages counts of *H. pylori* were $2.7x10^2$, $3.1x10^3$ and $2.2x10^2$ CFU/100ml, respectively. The presence of *H. pylori* was 33% in collected water samples using culture method. The presence of *H. pylori* in Nile River, El-Rahawy Drain and hospital wastewater, may be attributed to high infection prevalence of *H. pylori* in Egyptian people, which reached to 90% **(**WGO, 2010). Another, possible explanation is that El-Rahawy Drain receives large amount of primary treated wastewater from Zenin and secondary treated wastewater from Abou-Rawash wastewater treatment plant. Both treating the municipal wastewater of Greater Cairo then, discharging directly into Nile River (Rossita Branch). Moreover, *H. pylori* have the ability to convert itself from spiral shape to coccoid when reached to water for protection (Nilsson *et al.,* 2002). Several media have been used for the isolation of *H. pylori* which is not extremely fastidious, and very sensitive to oxygen, since it is a microaerophile, and requires an incubation period of 3 to 5 days (Goodwin and Armstrong, 1990). Columbia agar is considered suitable media for detection of *H. pylori.* Columbia agar as a base containing the antimicrobial of vancomycin, amphotericin B, trimethoprim and cefsulodin to inhibit contaminating flora without loss of recovery of *H. pylori* (Dent and McNulty, 1988). The cefsulodin concentration has been increased to provide improved inhibition of contaminating flora (Stevenson *et al.,* 2000).

There is clearly no one indicator that may be suitable for all pathogens for all aquatic environments (Yates, 2007). Correlation between the presence of bacterial indicators and the presence of pathogens were low (Savichtcheva and Okabe, 2006). As a result, none of the bacterial indicators currently used meets all established criteria for water quality. Thus in certain cases, such as drinking or bathing water, direct analysis of specific pathogens of concern is considered to be suitable alternative.

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