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ORIGINAL ARTICLE

Effect of dilution factor on the isolation of *Helicobacter pylori* from municipal wastewater using culture technique

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ABSTRACT

Background and Objectives: Isolating *Helicobacter pylori (H. pylori)* from wastewater and culturing it using a conventional method has always been a controversial issue because the bacterium converts into a coccoid form when exposed to an unfavourable environment like wastewater. To clarify the cultivability behaviour of the bacterium in fresh wastewater samples, the effect of municipal wastewater dilation on the cultivation of the bacterium using a conventional method was examined.

Materials and Methods: Several dilutions of wastewater samples were inoculated with fresh *H. pylori* suspension (with McFarland's dilution 0.5) to examine the dilution effect of wastewater on the bacterium isolation.

Results: The *H. pylori* growth was found to be possible for a dilution factor from $1/10^6$ to $1/10^7$ of raw wastewater. In higher dilution factors the growth of fungi was dominant and could prevent the isolation of the bacterium.

Conclusion: The optimized technique could be applied in future studies for increasing the chance of *H. pylori* isolation from fresh wastewater environments.

Keywords: *Helicobacter pylori*; Wastewater; Culture techniques

INTRODUCTION

Helicobacter pylori (H. pylori) is a Gram-negative pathogenic bacterium which is known to affect more than 50% of the world population (1, 2). *H. pylori* is recognized as the major cause of gastritis and peptic ulcer and gastric mucosa-associated lymphoid tissue (MALT) gastric lymphoma (3). There were approximately 4.4 billion individuals with *H. pylori* infection worldwide in 2015 (4). The infection prevalence is highest in Africa (79.1%), Latin America and the Caribbean (63.4%), and Asia (54.7%). Water supplies contaminated with faecal materials may be a potential source of *H. pylori* transmission (1). This

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is particularly relevant in developing countries where municipal wastewaters are not adequately treated and discharged directly or with partial treatment into receiving water bodies and rivers or other water resources (5). *H. pylori* is reported to be present in stools and aquatic environments with different levels of faecal pollution (6). There is a growing consensus that *H. pylori* is a waterborne pathogen. Moreover, the ability of this pathogen to survive with infectious virulence in the environment could not be easy to demonstrate because its recovery from aquatic environments is difficult (7). The polymerase chain reaction (PCR) assays and quantitative real-time PCR demonstrated that this pathogen can be present and viable in all processed wastewater samples in wastewater treatment plants (WWTP) (8).

In many countries, WWTP's effluent is increasingly reused for domestic, industrial, and agricultural purposes. Therefore, the presence of this bacterium in reclaimed waters can be a threat to public health (9). The presence of *H. pylori* cells in raw wastewater or reused wastewater is a possible reason why the pathogen re-enters the water or food chains, which is a public health concern. Therefore, the isolation of the bacterium from wastewater and clarification of the role of the wastewater environment on the transmission of this pathogen needs to be further examined. Attempts to isolate *H. pylori* cells from wastewater samples using culture technique have been made but have largely been unsuccessful or time-consuming and difficult (10, 11). Moreover, the ability of the bacterium to persist in a viable but non-culturable (VBNC) state is common (12). Therefore, in most cases, culture-independent techniques such as PCR and fluorescent in situ hybridization (FISH) (7) are being used to enable the monitoring of pathogens in water and wastewater (13). These techniques are relatively expensive methods to detect *H. pylori* in environmental samples. Therefore, culture techniques will be more practical both in terms of cost and routine use if they can be used appropriately for *H. pylori* isolation from wastewater.

WWTPs are considered hotspots for antibiotic-resistant genes and the spread of bacteria into the environment (14, 15). Therefore the presence of antibiotic-resistant bacteria in wastewaters can increase the potential risk of gene transfer to non-resistant bacteria in this media (16). The isolation of pure bacterial culture using culture-dependent methods is essential for the study of the pathogen virulence, its antibiotic susceptibility, and its genome sequence (17). The major advantage of culture-dependent techniques over molecular techniques lies in that they provide microbial material that can be used in further analysis (18). The culture-dependent methods allow not only for the isolation of a pure culture of bacteria and their identification, but also characterization of their morphology, biochemistry, and biology of them (19). But it is believed these methods fail to detect VBNC forms of pathogens like *H. pylori*. Alternatively, the PCR technique can be considered as either a method for detecting VBNC forms of pathogens but this molecular method is more expensive for routine work than the cultivation technique, and the laboratory must have appropriate equipment and experience.

Despite the disadvantages of culture techniques for the detection of VBNC forms of pathogens, this technique is the gold-standard method to detect viable *H. pylori* from environmental media when compared with molecular techniques. Furthermore, bacteria isolation is important for epidemiological and antimicrobial sensitivity analysis (20). Hortelano et al. (20) suggested the optimization of cultivations techniques is essential for the isolation of *H. pylori* from environmental samples. Therefore, if culture methods can be optimized in any way to isolate this bacterium, then it is possible to measure or detect the presence of bacteria in environmental samples using culture methods, which are cheaper compared to molecular techniques. On the other hand, the induction of the VBNC state and complete loss of culturability of bacteria in industrial samples is a long-term process, which might take up to months to occur (21). But exposure to heavy metals and applying chlorine treatment or UV light have been shown to reduce cultivability and stimulate VBNC induction (22, 23). In fresh municipal wastewater, the concentration of heavy metals and chlorine is not significant to stimulate VBNC induction rapidly. It is plausible to suggest that breaks in municipal water pipes allow for infiltration of contaminated surrounding waters (24), which may be contaminated with fresh sewages. Moreover, one of the potential problems in water supply systems especially in municipal and hospital settings is negative pressures when fresh wastewater or liquid from sewers, toilets, laboratory sterilizers and syphon apparatus backflow into water supply systems. Thus an urgent examination of wastewater or water samples using culture techniques may help to identify the source of such pollutions. As it

is believed that starvation is the important VBNC state-inducing factor for *H. pylori* (22, 25) and the isolation of *H. pylori* from wastewater is even more difficult due to the presence of contaminants (26), the wastewater dilution may be assumed as one of the factors increasing the chance of bacterial isolation from fresh wastewater. So far, few studies (20) have examined the effect of wastewater dilution on the isolation of bacteria using cultivation techniques from municipal wastewater.

In this study, several experiments were conducted to examine the effect of sample dilution on the isolation of *H. pylori* from fresh municipal wastewater using the culture technique. The study is going to highlight the limitation of the culture technique for the isolation of *H. pylori* from municipal wastewater and is showing the effect of wastewater dilution on increasing the chance of *H. pylori* isolation from raw wastewater.

MATERIALS AND METHODS

Isolation experiments. No standard method for the isolation of *H. pylori* from sewage has been introduced so far, thus we conducted the following experiments to examine if it is possible to isolate *H. pylori* using culture methods. In the first experiment direct filtration was used for the isolation of *H. pylori* from real municipal wastewater as follows:

Experiment 1: direct filtration. One litre of composite municipal sewage sample was collected from one of the biggest wastewater treatment plants in Tehran (Sharak Qarb WWTP). The sample was stored in a cold condition at about 4°C and transferred to the laboratory within less than one hour for further analysis. The sample container was well shaken and distributed in four sterile containers. The bacterial strain used in this study had previously been isolated from a gastroenteritis patient biopsy. The selected isolate had all genes including *cag* PAI, *vac*A (s1, m1) and *cag*A. The *cag*A and the *vac*A genes are major virulence factors in *H. pylori* responsible for gastric pathology (27). Moreover, in a study conducted by Lu et al. (11), the majority of *H. pylori* isolates from wastewater samples were of vacA/s1 and vacA/m1 types, which have been shown (28) to be associated with highly progressed diseases in gastric cancer patients.

One ml of *H. Pylori* suspension (with standard 0.5 McFarland) was added to two bottles. One of these bottles immediately and the second one after 24 hours of incubation at room temperature, were passed through 0.45 µm-pore-size nitrocellulose filters. Similarly, the other two bottles were filtered but without bacterial inoculation, one bottle immediately, and the other one after 24 hours at room temperature. Using vacuum filtration, the samples were concentrated on 0.45 µm filter. The filter papers were then placed on the specific culture medium of Brucella agar containing 10% of defibrinated sheep blood, 10% of beef embryo serum (FCS) and selective supplements of campylobacter (vancomycin, polymyxin and trimethoprim and amphotericin B) (Fig. 1). Cultured media were incubated in a microaerophilic atmosphere (5% O_2, 10% CO_2, 85% N_2) at 37°C for 1 hr. Then, filter papers were removed from the cultures and they were incubated at 37°C for 5 days.

Experiment 2: dilution and filtration. One another composite fresh sewage sample was collected using a sterile bottle from the inlet of Sharak Qarb WWTP. The sample was transferred to the microbiology laboratory within less than one hour to be used for further analysis. A dilution of 1/100 was prepared from the sample directly (Fig. 2) and transferred to two tubes. One ml of *H. pylori* suspension (with stan-

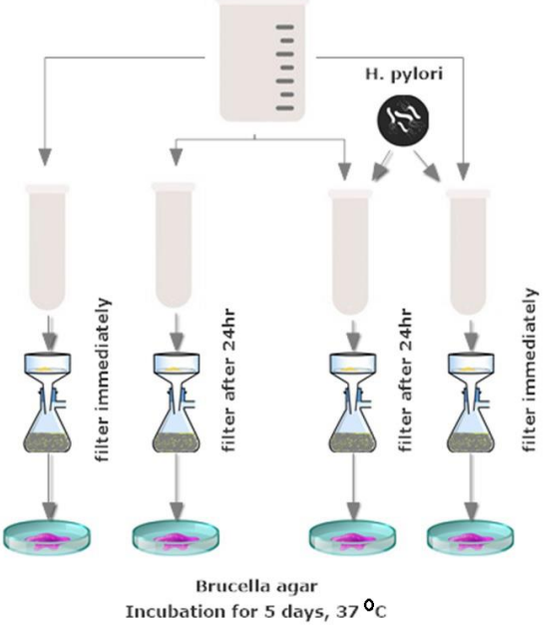

Fig. 1. Culturing wastewater sample on Brucella agar without dilution

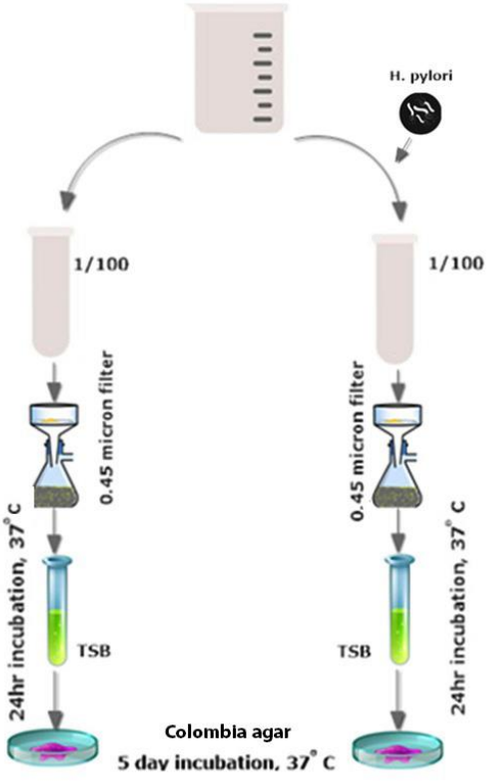

Fig. 2. Culturing wastewater sample on Colombia agar with dilutions 1/100

dard 0.5 McFarland) was added to one of the tubes. The samples in both tubes were filtered using 0.45 µm filter papers. The filter membranes were eluted in 2 ml tryptic soy broth (TSB) and incubated for 24 hours at 37ºC in a microaerophilic condition. After this period of incubation, one ml of the enriched solution was cultured on the Colombia agar medium containing 10% of defibrinated sheep blood, 10% of beef embryo serum (FCS) and selective supplements of campylobacter (vancomycin, polymyxin and trimethoprim and amphotericin B). The media were incubated at 37ºC in microaerophilic condition for 5 days.

Experiment 3: increasing dilution factor. In the third examination (Fig. 3.a), wastewater dilution factors were increased to examine their effect on reducing the interference effect of possibly toxic substances, fungi and other bacteria on the growth of *H. pylori*. In this trial, another fresh composite sample was collected again from the inlet of the wastewater treatment plant. The sample was transferred to the laboratory as mentioned in experiment 1. A dilution of 1/10 was prepared from the original sample and 1 ml of the diluted sample was further diluted with 9 ml of Muller Hinton Broth (MHB) medium. Serial dilutions 1/100 to 1/1000000 were prepared from the tube of 1/10 dilution (Fig. 3). One another 10 ml tube of Muller Hinton broth was used as the control tube. Using fresh cultured *H. pylori* colonies, a suspension with McFarland's dilution of 0.5 was prepared. 400 µl of McFarland's dilution 0.5 was transferred to each of the six diluted samples and the control tube as well. 1 ml of sample from each tube was inoculated on Colombia agar medium containing 10% of defibrinated sheep blood, 10% of beef embryo serum (FCS) and selective supplements of campylobacter (vancomycin, polymyxin and trimethoprim and amphotericin B). All cultured media were incubated at 37ºC in microaerophilic condition for 5 days.

In six other tubes (Fig. 3.b), serial dilutions of 1/10 to 1/1000000 were prepared from the diluted wastewater sample without inoculation with *H. pylori* suspension. 1 ml from each of these tubes was inoculated on Colombia agar medium containing 10% of defibrinated sheep blood, 10% of beef embryo serum (FCS) and selective supplements of campylobacter (vancomycin, polymyxin and trimethoprim and amphotericin B). All of these cultured media were incubated at 37ºC in microaerophilic conditions for 5 days.

The isolates which gave a positive result by biochemical tests as *H. pylori* were further confirmed

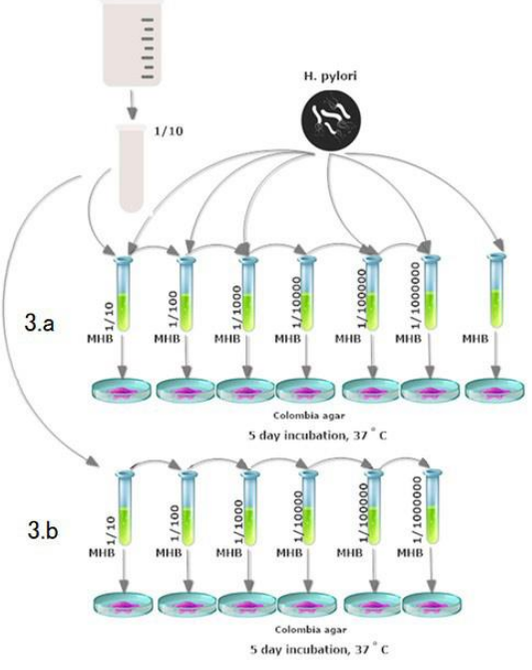

Fig. 3. Inoculation of fresh *H. pylori* suspension into several dilutions of wastewater

by PCR assay. The isolates were confirmed by using primer glmM for identification of *H. pylori* and based on 16S rRNA sequence. (29). The primer comprised of forward sequence: 5'-GGATAAGCTTTTAGGGGT-GTTAGGGG-3' and reverse: 5'- GCTTACTTTCTA-ACACTAACGCGC-3'. The genomic DNA of colonies was extracted by the method described in (30).

In this study some of the statistical analysis including Pearson's chi-squared test was applied to the results of the study conducted by Moreno et al. (31) to examine the independence of the PCR, fluorescent in situ hybridization (FISH) and Direct Viable Count Combined with FISH (DVC-FISH) techniques from culture technique.

RESULTS

Fig. 4. shows the culture plates obtained for the first and second experiments to examine the effect of direct filtration and filtration with dilution at 1/100 of the original concentration, respectively. As shown, the experiments could not provide successful results for isolating the bacterium from both inoculated and non-inoculated samples (see Table 1 and Fig. 4). Fig. 5 shows the culture plates for the third experiment of the inoculation of fresh *H. pylori* suspension into several dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 0^{-5}, 10^{-6}, and
10^{-7} of wastewater. As shown, significant growth for *H. pylori* was observed in Fig. 5 g-h. The results of this experiment were summarized in Table 1. The biochemical urease test for the observed colonies of *H. pylori* and the gel images of positive PCR amplicons were shown in Fig. 6 and Fig. 7, respectively. Table 2 shows the results of the statistical analysis of culture technique independence from PCR, FISH and DVC-FISH based on the results of the study conducted by Moreno et al. (31).

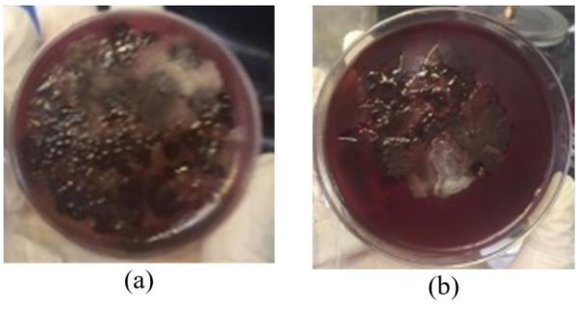

Fig. 4. Result of culturing wastewater sample on Colombia agar for (a) non-inoculated and (b) inoculated samples

DISCUSSION

H. pylori is mostly found in a spiral shape within the body of a human host, but it is prone to convert into a coccoid form when it is exposed to an unfavourable environment (32). In this study, we tried to examine the cultivability behaviour of *H. pylori* while exposing it to a wastewater environment with different dilutions. Several experiments were conducted to examine the isolation of the bacterium from municipal wastewater.

As shown in Fig. 4, the results of the first and second experiments namely direct filtration and filtration along with dilution at 1/100 of the original concentration were not successful for isolating the bacterium from both inoculated and non-inoculated samples. In these two experiments in all the plates, the growth of fungi was found to be prominent. In the third experiment (Fig. 3/3.a, Table 1), after the incubation period and opening in the jars as presented in Fig. 5, the plates showed a decreasing trend in the growth of fungi from the dilution factors of 1/100 to 1/10000. For these dilutions a bacterium resembled to be *H. pylori* was not observed. But for the dilutions of 1/1000000 and 1/10000000, a significant growth for *H. pylori* was observed (Fig. 5 g-h). The observed colonies of *H. pylori* were confirmed by biochemical urease test (Fig. 6). PCR test also further confirmed the isolates of *H. pylori* (See Fig. 6). This experiment show that the lower the dilution of the wastewater, probably the lower the dilution of toxic and interfering substances which could prevent the bacterium to growth. The result of this experiment recommends that the dilution factors of $1/10^6$ to $1/10^7$ could minimize the probable effects of interfering substances which may be present in fresh wastewater on the growth of *H. pylori*.

Adams et al. (25) suggest that *H. pylori* can remain culturable in natural waters for 2 to 3 days when the waters are at a low temperature and the bacterial cells exist in all morphologies in the culturable and nonculturable states. They also believed the most important VBNC-inducing factor for *H. pylori* is the drop in nutrients or starvation (22, 25). Therefore, it seems that the cultivation techniques may be applied with sufficient dilutions for the isolation of *H. pylori* from fresh sewages in which the bacterium has likely not had enough time to enter the VBNC state. Moreover, Robben et al. (33) also showed that even with prolonged exposure of Gram-negative bacteria

Table 1. Results of experiments on *H. pylori* culturing in the presence of wastewater

Experiment	Sample Dilution ratio	Inoculation	Room incubation	Filtration	Pre-incubation	Culturing/final incubation	*H. pylori* isolation
Experiment 1:	1	Yes	No	0.45 µm filter	No	Brucella agar,	No
direct filtration	1	Yes	Yes (24 hr 25ºC)			37ºC, 5 days	
	1	No	No				
	1	No	Yes (24 hr 25ºC)				
Experiment 2:	$1/10^1$	No	No	0.45 µm filter	TSB, 37ºC,	Colombia agar,	No
dilution and filtration	$1/10^2$	No	No		24 hr	37ºC, 5 days	
Experiment 3	$1/10^3$	No	No				
(a): Culturing with *H. pylori* inoculation	$1/10^2$	Yes	No	No	MHB, 37ºC,	Colombia agar,	No
	$1/10^3$	Yes	No	No	24 hr	37ºC, 5 days	No
	$1/10^4$	Yes	No	No			No
	$1/10^5$	Yes	No	No			No
	$1/10^6$	Yes	No	No			Yes
	$1/10^7$	Yes	No	No			Yes
Experiment 3 (b): Culturing without *H. pylori* inoculation	$1/10^2$	No	No	No	MHB, 37ºC,	Colombia agar,	No
	$1/10^3$	No	No	No	24 hr	37ºC, 5 days	No
	$1/10^4$	No	No	No			No
	$1/10^5$	No	No	No			No
	$1/10^6$	No	No	No			No
	$1/10^7$	No	No	No			No

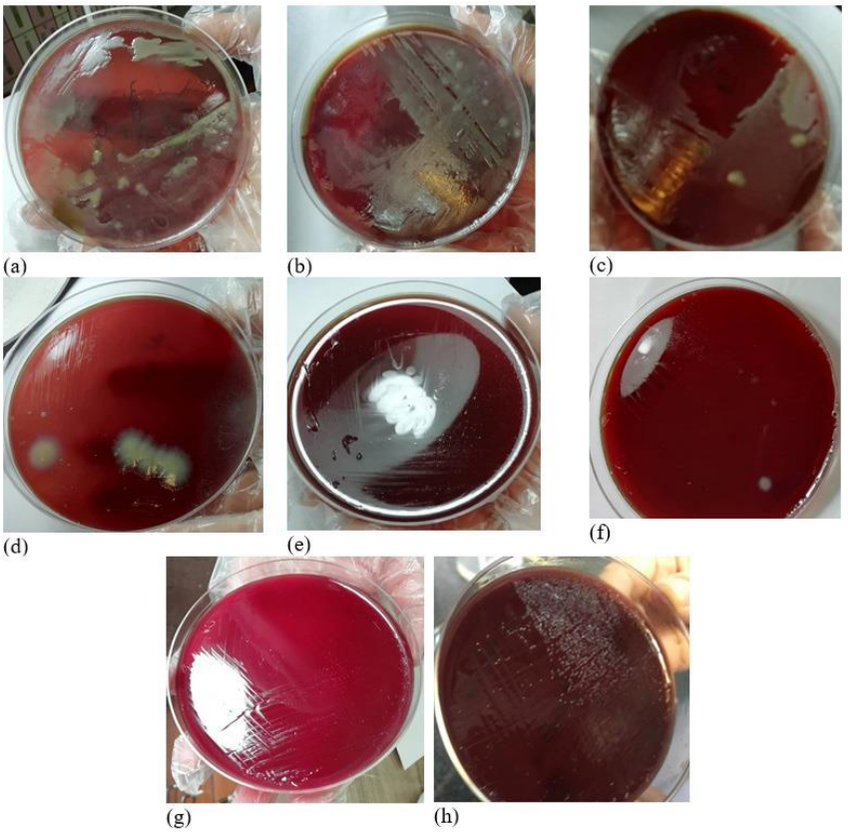

Fig. 5. Inoculation of fresh *H. pylori* suspension into several dilutions of (a) 10^{-1}, (b) 10^{-2}, (d) 10^{-3}, (e) 10^{-4}, (f) 10^{-5}, (g) 10^{-6}, (h) 10^{-7} of wastewater

Fig. 6. Urease test, yellow: negative, purple: positive

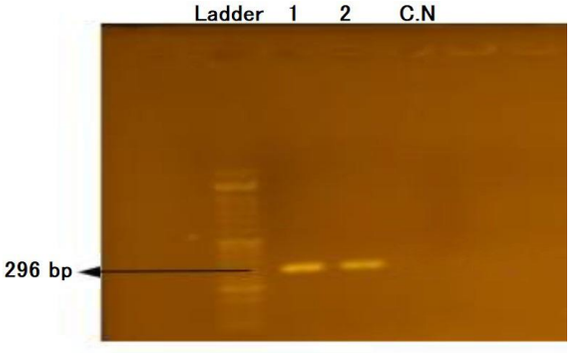

Fig. 7. Diagnosis of *Helicobacter pylori* PCR for glmM gene (length 296 bp) extracted from *H. pylori* colonies on the plates with dilutions $1/10^6$ (1) and $1/10^7$ (2); C.N is negative PCR control

Table 2. The independence of culture technique from PCR, FISH and DVC-FISH based on results of the study conducted by Moreno et al. (31)

Comparison	Contingency coefficient	p-value
Culture/PCR	0.171	0.2296
Culture/FISH	0.139	0.3323
Culture/DVC-FISH	0.447	0.0005

to surfactant and salt combinations for 24, the cells often could not enter the VBNC state. Thus, they believed the induction of Gram-negative bacteria to enter the VBNC state is a dynamic process that is not completely predictable. It is because they possess a second cell membrane that serves as an additional barrier to surfactants and salts in comparison with Gram-positive bacteria.

In the case of tubes cultured without *H. pylori* inoculation (Fig. 3/3.b), no specific isolates were observed in all of the culture media. This observation may be due to the absence of bacterium in the real wastewater sample. However, for further investigation, two more samples of municipal wastewater were taken and cultured by applying the obtained optimal dilution factors of $1/10^6$ to $1/10^7$. Furthermore, a PCR test was also conducted to evaluate the presence of the pathogen in the samples. The result of the PCR test was negative for all samples and the results of culture experiments in serial dilutions of wastewater were also found to be negative. Lu et al. (11) reported the isolation of *H. pylori* cells from raw municipal wastewater taken in Mexico, employing also an immunomagnetic separation technique before culture. They believed their success in the separation of *H. pylori* may have been due to the high *H. pylori* prevalence rate in the study area (74%), which increased the likelihood that the cells were introduced into the wastewater shortly before the isolation was made. Moreover, they believed the low temperature during the sampling season had been favourable for the bacteria to stay in a culturable status. This temperature-culturability relationship was further suggested in (34) when *H. pylori* cells were observed to be remained culturable in nutrient media incubated at 4°C in comparison with the results seen at 25, 40, and 42°C. Adams et al. (25) also showed that *H. pylori* cells can remain culturable longer in cooler waters than in warmer (>20°) waters. Thus some of the reasons that *H. pylori* was found in none of our samples may be the low prevalence rate of the pathogen in the study area and probably the increase in the temperature of the samples in laboratory during the analysis.

According to the results of this study, dilution of sewage samples can increase the possibility and probability of culturing and then the isolation of *H. pylori* bacteria from sewage. But it should be noted that in this study the sewage samples were inoculated with fresh bacteria, so in any cases where the pollution (the entrance of *H. pylori* into wastewater) has occurred newly, there is a more probability of isolation of bacteria by culture method. This is because the bacterium may have not enough time to convert into VBNC form. In a VBNC state, the cells typically demonstrate very low levels of metabolic activity (35-37). Adams et al. (25) demonstrated that *H. pylori* can enter into the VBNC state in which cells were observed to become non-culturable in freshwater microcosms. Moreover, many suggest that *H. pylori* persist in the environment in a VBNC form (38-40).

The optimal wastewater dilution factors found in this study can be examined in future studies to improve the environmental condition for the isolation of the bacterium from wastewater samples. However, it should be noted that there are several difficulties in isolating *H. pylori* from wastewater using the culture technique. For example, Adams et al. (25) summarized these difficulties as the low sensitivity of the culture method for the detection of *H. pylori* by factors such as a small number of pathogens present in the samples, the death of the bacterium during the manipulation of the samples, and also the conversion of bacterium into the VBNC status. In general, there are still many controversies regarding the use of the culture method to isolate this bacterium from wastewater. But it does not mean that *H. pylori* could not be isolated from wastewater at all, because in most of the cases where unsuccessful results were obtained, the massive growth of competitive biota like fungi in selective media, as seen in this study for dilution factors of 1/100 to 1/100000, is one of the main challenges for isolation of *H. pylori* from water environments (41, 42). Moreover for environmental water samples like wastewater due to the unavailability of adequate transport media and the presence of interfering substances and contaminants, the isolation of *H. pylori* is more difficult in comparison with clinical samples (26). The detection of the pathogen in wastewater samples mostly depends on the enrichment of culture to stimulate the growth of *H. pylori* and to inhibit the growth of other competitor microorganisms (43). Azevedo et al. (37) noticed that the media which are too rich in nutrients may also cause a nutritional shock and prevent the growth of the bacteria. Thus, some of the authors are focused on evaluating different types of culture media for the detection and quantification of *H. pylori* in environmental samples (20). Besides the modifications in culture media, selective separation techniques like immunomagnetic beads for selective isolation of *H. pylori* were also applied based on the immunological properties of the bacterium. In a study conducted by Lu et al. (11) for the isolation of *H. pylori* from municipal wastewater, several samples with a dilution factor of 1/100 were filtered using vacuum filtration on a nitrocellulose filter. The filters were eluted then into tryptic soy broth containing antibiotics and incubated at 37°C for 24 h in a microaerophilic condition. These enriched cultures were then further processed in an immunomagnetic separation (IMS) technique. The study suggested the IMS as an important initial concentration step could not only be selective for *H. pylori* but also can eliminate possible contaminating substances that may interfere with culturing.

Along with the modifications have been on culture techniques (20), culture-independent molecular techniques such as PCR (31, 44), FISH (7, 31) and DVC-FISH (31, 45) are used for the detection of *H. pylori* in environmental water samples. In a study conducted by Moreno et al. (31) several methods including PCR, FISH and DVC-FISH were applied directly for the analysis *H. pylori* in wastewater samples. The study aimed to determine the presence of cultivable and therefore viable *H. pylori* in wastewater treatment plants. To compare applied methods or examine the relationship amongst them we tried to assess the correlation amongst culture technique, PCR, FISH and DVC-FISH by statistically analyzing the data presented in Moreno et al. (31). The p-value for Pearson's chi-squared test and relevant contingency coefficient for each comparison are summarized in Table 2. As summarized, just only a significant correlation (p-value<0.05) was observed between the results of the culture technique and DVC-FISH. No significant correlation (p-value>0.05) was observed between the results of the culture and PCR technique. The finding is consistent with the study of Queralt et al. (38) who claim the PCR technique appears to overestimate the presence *H. pylori* in samples while the culture technique on contrary could underestimate the presence of the pathogen. Such underestimation may be due to the presence of viable but non-culturable (VBNC) *H. pylori* in the analyzed samples. On the other hand, the significant correlation observed between culture technique and DVC-FISH suggests that DVC-FISH may also underestimate the pathogen presence and probably may not be an appropriate technique for detecting all VBNC cells within the samples. In overall, given the fact that these VBNC bacteria can maintain their pathogenicity virulence, and they could be considered as a potential thread for infection transmission (46), the application of PCR which is more sensitive technique could be considered as the standard technique when and where the detection of the bacterium regarding to its health concerns is important.

The obtained optimal dilutions found in our study may reduce the effects of interfering substances and increase the chance of the bacterium isolation for further analysis.

CONCLUSION

Although there is no established culture technique for the isolation of *H. pylori* from the wastewater, the results of this study showed that dilution of wastewater samples can increase the chance of identification and isolation of the pathogen from wastewater. Dilution of wastewater samples can be especially effective on newly contaminated samples. However, to better evaluate the results of dilution factors on the isolation of bacterium from wastewater, it is recommended to perform the similar optimal experiment found in this study, on a relatively large sample size of municipal wastewater samples to investigate the effect of sample dilution on *H. pylori* isolation from wastewater. It seems, due to the presence of the VBNC coccoid form of the bacterium in wastewater, PCR could be a great tool to detect the bacterium in wastewater samples. So, it is recommended to examine the correlation between the results of PCR tests and the results obtained from the culture technique in future studies with a relatively large sample size.

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