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Persistence of *Helicobacter pylori* Coccoid forms in Different Environments

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Abstract

Objective: The present study aims to determine the possibility of *H. pylori* coccoid forms to survive in our surrounding environments, thus exhibiting public health hazard.

Methods: *Helicobacter pylori* strain Makkah 7 accession number HQ622108, was inoculated in samples of soil, well, tap, swimming pool, and sewage water; in which, an average initial inoculum of 10^7 CFU/ml was inoculated. The experiment was conducted, outdoor where ambient temperature varied between 39–55°C in Jeddah city, Saudi Arabia. The survival rate of *H. pylori* strain was quantitatively and qualitatively studied for five months.

Results: Viable counts were undetectable after 24 hours, while total count varied between 10^7 to 10^6, and declined to 10^3 bacterial cell/ml five months later. Interestingly, microscopic examination revealed few non-motile rods and motile coccoid forms. All samples were removed and placed at 4°C for one month. Following, total count, morphology, and motility were examined, showing motile coccoid forms. Electron microscopic and, molecular studies were carried out, confirming that the species is *H. pylori,* which was detected by using 16S rRNA primer for *H. pylori* with a product size of 163 bp. The ability of *H. pylori* strain to persist and survive, by being motile in the coccoid form, for five months under such hostile environment, strongly indicates that the coccoid form plays a vital role in the transmission, recrudescence, and therapeutic failure. It is indeed a hazardous and a crucial infectious phase of this bacterium.

Conclusion: Detecting coccoid forms in water and soil, accompanied by their eradication, must be seriously considered and applied. Consequently, hindering and preventing different diseases caused by *H. pylori.*

Keywords: *Helicobacter pylori,* coccoid forms, survival, count, environments

Introduction

H. pylori is a motile, non-sporing, Gram negative spiral, rod, S-shaped, and a microaerophilic fastidious bacterium, with distinctive coccoid-form state. It was discovered by Warren and Marshall in 1982, proving that it is the aetiological agent of type b gastritis as well as gastric carcinoma and MALT Lymphoma.[1] Surprisingly, the human Gastric mucosa is considered as the natural habitat of this amazing bacterial species. Paradoxically however, was identified as class one carcinogen.[2,3] Nonetheless, many subjects may harbor *H. pylori* as asymptomatic carriers for a lifetime; bearing in mind, that *H. pylori* strain variations impact its virulence.[4,5] Eventually, research flourished and revealed a vast amount of knowledge, that favored many patients complaining of gastrointestinal disorders, as well as attracting interest of academic investigators.[6–14]

On the other hand, accumulated data concerning *H. pylori* therapeutic failure, is commonly attributed to antimicrobial resistance.[15–17] Regrettably, the masterpiece of *H. pylori* bacterium namely the coccoid form, is grossly neglected. The latter, is the converted spiral phase when exposed to detrimental conditions. It is extremely crucial since it is capable of conquering any harsh environment, including antibiotics. As documented, two main phases of coccoid forms are known, the non-viable dying phase, and the so-called viable but unculturable phase.[18] The unfavorable conditions that induce the spiral forms to convert into the coccoid forms, include increased oxygen tension, temperature, pH, depletion of nutrients, prolonged incubation, and antimicrobial drugs. Nevertheless, the converted coccoid forms are in turn, capable of reversing in vivo into the spiral form.[19,20] Interestingly, some authorities in the field, demonstrated the existence of both forms in damaged gastric mucosa, where coccoid forms were more abundant in adenocarcinoma lesions.[21] Thereby, coccoid forms may be incriminated in therapeutic failure, recrudescence, as well as constituting a source of infection, and other disorders, since they harbor many virulence factors.[22–24] Those factors include bacterial colonization factors such as BabA, SabA, OipA, and HopQ. Whereas, factors that are necessary for gastric pathogenicity, include the effector proteins such as VacA, HtrA, and CagA. The latter polymorphic gene, was found to be involved in human cancer.[25,26] Additionally, and certainly, biofilm formation could never be ignored in the process of *H. pylori* pathogenesis.[19,27]

Furthermore, recent evidence suggests that drinking water, freshwater stream, wastewater, surface water, and groundwater, can be potential sources of *H. pylori*, thus some researchers stressed upon improving detection methods, in various water types.[9,28,29] Similarly, using molecular methods, El-Sharouny et al. (2015)[30] detected DNA of *H. pylori* in two water samples, and emphasized the possibility of *H. pylori* to be transmitted via drinking water; hence acting as a risk factor for outbreaks of infection, particularly in dense populated areas. What's more, Atapoor et al., 2014[31], reported that out of 460 samples (9.56%) of un-washed vegetable salads were positive for *H. pylori* using the culture method, while, 10.86% were positive using polymerase chain reaction technique. Moreover, cumulative studies have attributed route of transmission, to faecal-oral route, person-to-person, and, contaminated water.[32,33] Taking the above data into consideration, the present work was an attempt to determine the possibility of coccoid forms to survive in our surrounding environments, such as different types of water and soil. Obviously, these surroundings could be emphasized as a risk factor, a source, and a route of transmission of *H. pylori* infections.

Materials and Methods

Bacterial Strain

Vaculating cytotoxin A positive strain of *H. pylori* was used in the present study ("Makkah 7" strain, accession number HQ 622108) which had been isolated from a gastric biopsy of a patient suffering from chronic active gastritis.[10,22] The strain was grown on blood agar under microaerophilic conditions at 37°C for five days. Also, a prepared wet specimen of *H. pylori* colonies was examined by the contrast microscopy to check motility.

Water and Soil Sample Collection and Processing

Water samples (1000 ml) each, from tap water, well water, sewage water and a private swimming pool water were collected from Jeddah city in a sterile container. Desert, and fertile soil were both collected in sterile plastic bags from west and south Makkah Almokarramah region, Saudi Arabia. All collected samples were transferred directly and preserved at 4°C until used. Water samples were autoclaved, and filtered using 0.45 µm while the soil was sieved, washed with sterile distilled water several times, and autoclaved.

Preparation of the Bacterial Inoculum

The *H. pylori* strain was grown on seven plates of sheep blood agar under microaerophilic conditions, and incubated for five days at 37°C. The initial inoculum was taken from the harvested bacteria and serial dilutions were prepared to achieve an inoculum size of around 7.5 x 10^7 CFU/ml.[9]

Inoculation of Water and Soil Samples

Each 50-ml conical flask was filled with either 30 ml of each water sample or 3 g of each soil sample. All flasks with water samples were inoculated with the bacterial suspension to obtain 7.5. x 10^7 CFU/ml.[9] The soil sample was suspended in 3 ml water and inoculated with the bacterial suspension to achieve 7.5 x 10^7 CFU/g. In addition, flasks containing different samples without bacterial inoculation were used as a negative control. Afterwards, all flasks were kept outdoor at 39–55°C for five months during summer, at Jeddah city, Saudi Arabia. Readings were taken simultaneously every 24 hours, from both the control and the inoculated soil and water. The survival rate of *H. pylori* strains was quantitatively studied by culturing the bacteria from the previous environments on blood agar to estimate viable counts for 24 hours only, since no viable bacteria could be detected thereafter. Five months later, all specimens were removed from the natural climate and placed in a refrigerator at 4°C for one month. After which, total counts, morphology, and motility were restudied. Counting chamber, was used to obtain total counts, throughout the whole period of the experiment.[34] Identification of *H. pylori* was performed by morphological and biochemical studies including Gram staining, urease, oxidase and catalase tests for the isolates, whereas, molecular studies was achieved by using 16S rRNA primer for *H. pylori* with a MS163 bp.[10,22] In addition, motility was examined by using phase contrast microscope, to detect the characteristic motility of *H. pylori*.[35]

Molecular Studies, DNA Extraction and PCR Amplification

Centrifugation of the samples were carried out at 12,000 rpm for 5 min and the pellets were then collected. DNA extraction was carried out according to Williams et al. (1990)[36], amplified and PCR was performed. The PCR amplified product was analyzed using 1.2% agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light.[10,22] The size of the 16S rRNA fragment of 163 bp was estimated based on Gene Ruler™ 50 bp DNA Ladder (Fermentas GmbH, Germany).

Electron Microscope Examination

Bacteria were harvested from the culture and fixed in 2.5% glutaraldehyde, dehydrated, and examined using Formvar-coal coated grids and contrasted with phosphotungstic acid 2%, pH 7.0.[37]

Results

The resulted colonies of *H. pylori* grown on blood agar were examined under scanning electron microscope. Typical shape of *H. pylori* appeared as curved rod (Figure 1A & 1B). Gram stain of the *H. pylori* strain under investigations, revealed Gram negative rods, S-shaped and curved forms, before converting to coccoid forms. Urease, oxidase and catalase tests were vigorously positive. The prepared wet specimen of *H. pylori* colonies examined by the contrast microscopy, showed the characteristic motility which appeared as a rapid corkscrew motion of the bacterium.

The harvested bacterial cells were inoculated in soil and water. The total bacterial counts were daily recorded and results were compared and summarized in Tables 1 and 2. The pH of tap and swimming pool water was 7, while well water and sewage was 6 and 4 respectively. Total counts of well water sample ranged from 10^6 to 10^4 bacterial cell/ml within five months; tap water was from 10^7 to 10^5 bacterial cell/ml, swimming pool declined from 10^6 to 10^3 bacterial cell/ml, and sewage was from 10^7 to 10^6 bacterial cell/ml. Concerning the cell morphology and motility during the first month, all samples showed motile coccoid and motile rod forms except for swimming pool water which showed motile coccoid and immotile rods. Up to five months, all water samples showed motile coccoid forms, except sewage sample which showed motile rods within the second month. The measured soil pH was 6.5 and 8.0 for fertile and desert soil, respectively. The total bacterial counts in fertile soil ranged from 10^7 to 10^5, while in desert soil, declined from 10^7 to 10^3 bacterial cell/g after 5 months outdoor incubation. Motile coccoid forms were observed in fertile soil during the whole five months. On the other hand, immotile rods appeared only at the first and second month. Desert sample however, revealed immotile coccoid forms except for the first month, where they appeared as slow motile coccoid, while rods were immotile (Table 2).

The PCR amplification revealed the presence of the expected product size with 163 bp represented the 16S rRNA gene in all water samples as shown in Figure 2. Likewise, the fragment of 163 bp was also detected in fertile and desert soil (Figure 3). *H. pylori* which was grown outdoor in different water and soil samples under temperatures, ranging from 39–55°C, showed different morphologies when examined by the transmission electron micrograph. Coccoid forms dominated except in two occasions, where flagellated rods emerged i.e. in tap water after 15 days, and in sewage after seven days (Figures 4–9).

Table 1. **Persistence and average total count (Bacterial cell/ml) of *H. pylori* in different types of water for 5 months**

Months	Temp.	Total count				Morphology and Motility			
		Well water	Tap water	Swim. pool water	Sewage	Well water	Tap water	Swim. pool water	Sewage
1st	39°C–50°C	10^6	10^7	10^6	10^7	C + R +	C + R +	C + R –	C + R +
2nd	55°C	10^6	10^6	10^5	10^7	C +	C +	C +	C + R +
3rd	55°C	10^5	10^5	10^4	10^6	C +	C +	C +	C +
4th	45°C	10^5	10^5	10^4	10^6	C +	C +	C +	C +
5th	45°C	10^4	10^5	10^3	106	C +	C +	C +	C +

Swim. Pool.: Swimming pool Water, C: coccoid, R: rod, +: motile, –: non-motile.

Table 2. **Persistence and average total count (Bacterial cell/ml) of *H. pylori* in fertile and desert soil for 5 months**

Months	Temperature	Total count		Morphology and Motility	
		Fertile	Desert	Fertile	Desert
1st	39°C–50°C	10^7	10^7	C + R –	C + R –
2nd	55°C	10^7	10^6	C + R –	C –
3rd	55°C	10^6	10^6	C +	C –
4th	45°C	10^6	10^4	C +	C –
5th	45°C	10^5	10^3	C +	C –

C: coccoid, R: rod, +: motile, –: non-motile.

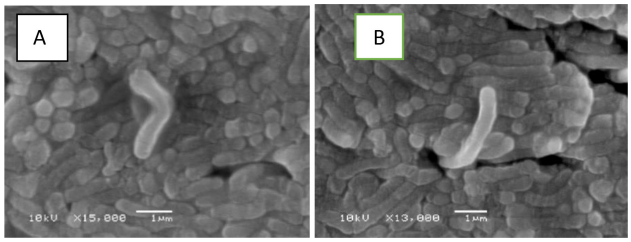

Fig. 1 **Scanning Electron Microscope of *H. pylori* grown on blood Agar, x15000(A) and x 13000 (B).**

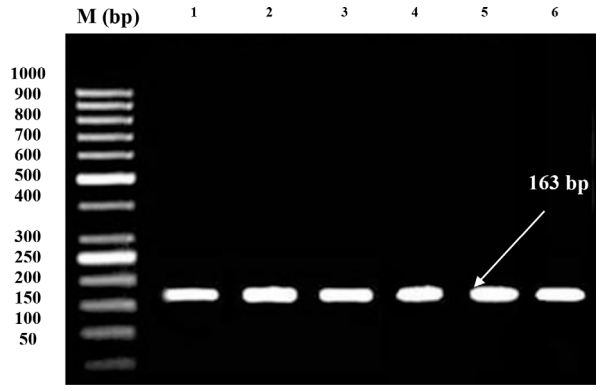

Fig. 2 **PCR amplified products of 16S rRNA gene of the *Helicobacter pylori* strain inoculated in well, tap, sewage, and swimming pool water using the designed primer with an expected size 163 bp. M = 50 bp DNA ladder.**

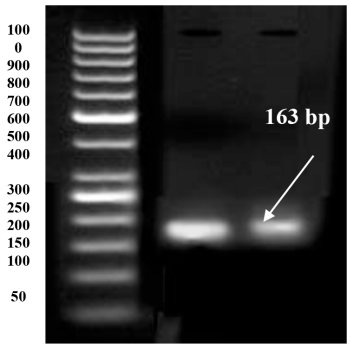

Fig. 3 **PCR amplified products of 16S rRNA gene of *Helicobacter pylori* strain inoculated in fertile soil and desert soil using the designed primer with an expected size 163 bp. M = 50 bp DNA ladder.**

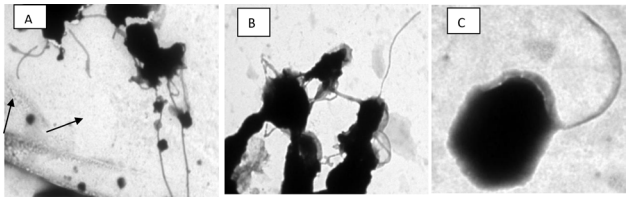

Fig. 4 **Transmission electron micrograph of *Helicobacter pylori* grown outdoor in well water, showing flagella with terminal bulb (arrows) after one week (A), showing coccoid and rod forms with flagella after two weeks (B) and showing coccoid form with flagellum after one month (C).**

Discussion

Environments have always played an important and significant role in dominating the nature of living organisms. The present study investigated the effect of hostile environments on the survival of *H. pylori* which revealed interesting and unexpected results. As known, how fastidious this bacterium is, and how difficult to grow, astonishingly yet, managed to thrive for a long period that would allow a global outbreak of infection in an instant. The magic that was granted to *H. pylori* is the master piece or the truly sovereign phase namely the coccoid form; which is without, could never persist and propagate. As reported in our results, *H. pylori* was found to persist in tap and sewage water for two weeks in the flagellated spiral form which is highly pathogenic (Figure 5A and 7A) and up to one month, in the coccoid form, as detected by electron microscopy and molecular studies. Obviously, this could be attributed to the harsh environment that it was preserved within, i.e. high temperature (39–55°C) and nutritional deprivation. In contrast, in a previous study, the survival of *H. pylori* strain in Jeddah tap water, maintained a population level of 5 x 10^4 CFU/ml, for 48 hours at room temperature. Moreover, the same strain persisted for five days at 4°C but declined from 5.33 x 10^4 to 8.75 x 10^2 CFU/ml in the fifth day. Furthermore, another strain at the same temperature, survived for up to eight days in Jeddah tap water, reaching viable counts of 3.88 x 10^3 CFU/ml, after which completely dropped to undetectable level.[9] Also, Tompkins et al. 1998[38], Karim and Maxwell 1998[39], and Sato et al. 1999[40], reported the survival and longevity of different *H. pylori* strains in various fluids from six to up to 16 days, depending on the initial inoculum. Thus, strain variations appear to influence the ability of survival under various conditions, where they still retain their pathogenicity.[11,41] Alternately, Hasanvand et al. (2023)[42] found three *H. pylori* positive water samples out of 50 tap water samples; and reported, that *H. pylori* was cultured from water and was capable of adapting to the stressed environment through conversion from the spiral to the coccoid form. Hence, they stressed upon tap water distribution systems as a potential route for *H. pylori* transmission.[43] Also, Bahrami et al. (2013)[44] noted that 4% out of 50 tap water samples, 5.8% out of 35 dental units' water samples, and 2.5% out of 40 samples, from water cooler in public places were found to be contaminated with *H. pylori*. In addition, as early as 1999, Hegarty et al.[45] demonstrated the correlation between stomach ulcers and contaminated well water in Pennsylvania, USA. Thus, confirming the ability of this bacterium to thrive in different types of water, for a sufficient period of time to induce infection by both forms. Indeed, literature is condensed with documents emphasizing that water plays a major role in the transmission of this organism.[46,47] Yet, acceptance and approval of water as a route of transference has not been strictly documented, and sadly remains indefinite.

Alternatively, our studies proved that soil, is another bank for restoring *H. pylori*. Though, desert soil was less supportive compared to the fertile soil; in which flagellated coccoid forms appeared in the latter, whereas,

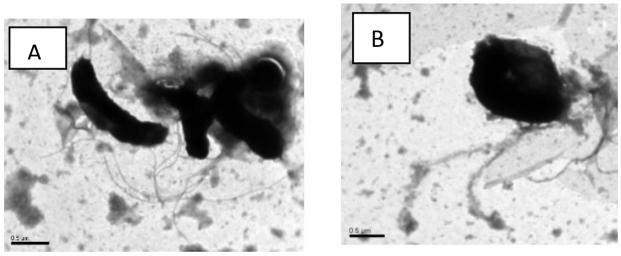

Fig. 5 **Transmission electron micrograph of *H. pylori* grown in tap water showing typical flagellated rod after 15 days of inoculation (A) and showing flagellated coccoid form after one month (B).**

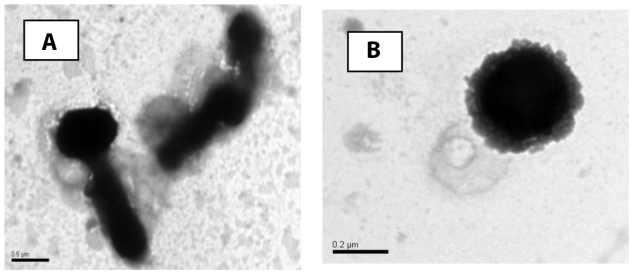

Fig. 6 **Transmission electron micrograph of *H. pylori* inoculated in swimming pool water showing flagellated rod after 15 days (A), coccoid form with rough surface and coiled flagella after one month (B).**

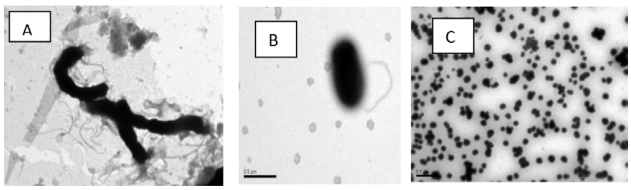

Fig. 7 **Transmission electron micrograph of *H. Pylori* inoculated in sewage water showing curved and rod forms with terminal bulb flagella after one week (A), showing short rod with flagellum after two weeks (B), and coccoid form after one month (C).**

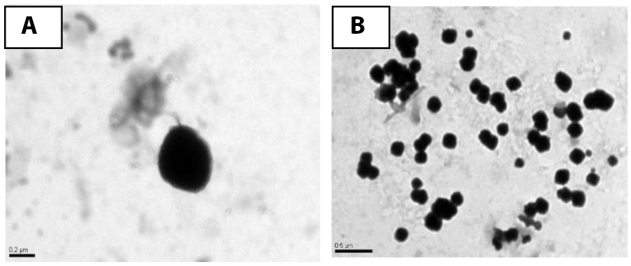

Fig. 8 **Transmission electron micrograph of *H. pylori* in fertile soil, showing coccoid form with flagellum after two weeks (A) and coccoid forms after one month of inoculation (B).**

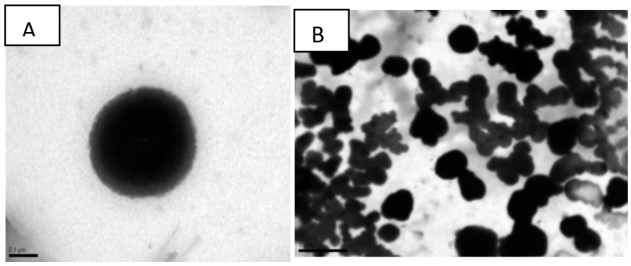

Fig. 9 **Transmission electron micrograph of *H. pylori* inoculated in autoclaved desert soil showing a single coccoid form after two weeks (A) and aggregates of coccoid forms after one month (B).**

clusters of coccoid forms were apparent in the former (desert soil). Whatever the forms are, coccoids are known to be pathogenic and incriminated in the transmission of *H. pylori*.[10,22–24] What's more, Poursina et al. 2013,[48] determined that coccoid forms possess virulence genes and contribute to chronic and severe gastric diseases. Likewise, Sasaki et al. 1999,[49] managed to detect DNA of *H. pylori* in field soil, water, flies, and cow faeces by nested polymerase chain reaction, and pointed out, that their existence in the environment acts as a probable route of transmission. In fact, few studies and interests are actually focused on coccoid forms, despite their numerous detrimental consequences in gastrointestinal disorders.[50] Above all, gastroenterologists must perceive the nature of coccoid forms, and develop specific strategies for their prevention and eradication. Further, thorough comprehensive research is desperately needed to overcome the in vitro and in vivo detrimental consequences of coccoid forms. Based on the entire previous data, emphasis on hygiene, appropriate sanitation, and public awareness, must be acknowledged to minimize the countless risks of *H. pylori* and their coccoids.

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Conflict of Interest

None.

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