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Detection of *Helicobacter pylori* and its associated host signals *in vitro*

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Abstract: Background: *Helicobacter pylori (H. pylori)* is one of the most common pathogens that can colonize and present in human gastric mucosa causing various gastrointestinal diseases. Methods: we were interested to determine the presence of *H. pylori* in the native-collection tap water that was contaminated with sewage by polymerase chain reaction (PCR) using specific primer for *H. pylori* – 16SrRNA. Further, quantitative real time- PCR (qRT-PCR) was used for the detection of *H. pylori* replication in HeLa cells using specific oligonucleotides for its 16SrRNA indicated by the relative gene expression. Results: Our results showed that most of the collected tap water samples revealed the same amplified fragment of 16SrRNA and the same fingerprint in PCR assay when compared with genomic DNA isolated from standard *H. Pylori* strain. Furthermore, the *in vitro* studying of *H. pylori* infection in HeLa cells showed high production levels of lactate dehydrogenase (LDH) from infected cells indicating the cytotoxic effect of *H. pylori* infection. Additionally, the relative expression of the Raf-1, autophagy-related Atg5, and PI3K were markedly increased in response to *H. pylori* infection in HeLa cells. Furthermore, *H. pylori* infection showed an obvious regulation of the production of the pro-inflammatory cytokines including IL-6 and TNF-α in a time-dependent manner. Conclusion: our data here provide sufficient evidences for the detection of *H. pylori* particles either in obtained samples or within the infected cell lines and highlight the potential cell signaling associated with *H. pylori* replication *in vitro*.

Keywords: Helicobacter pylori, molecular diagnostic, HeLa cells, cell signaling, cytokines.

1. Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium that colonizes the gastric mucosa and infects more than 50% of the world's population [1]. Long term *H. pylori* infection leads to serious injury in the gastric mucosa causing a variety of associated syndromes, such as chronic gastritis, peptic ulcer, gastric cancer, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [2]. Usually, *H. pylori* is transmitted person-to-person by saliva or by fecal-contaminated food and water. Noteworthy, in the developing countries, *H. pylori* prevalence occurred during childhood by a combination of several factors including contaminated water, crowded conditions, and poor hygiene [3,4]. Several diagnostic tests are used to determine *H. pylori* infection, including blood test, breath test, stool

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test, and scope test. Analysis of a blood sample may reveal evidence of an active or previous *H. pylori* infection. However, breath and stool tests are more accurate for detecting active *H. pylori* infections than a blood test. During a breath test, the tagged carbon molecules are used to swallow which releases detectable carbon molecules in the presence of *H. pylori* in the stomach. The stool test is an antigen test based on the interaction between specific antigen and associated *H. pylori* protein in the stool. The scope test is known as an upper endoscopy exam required sedated-patients to use endoscope which facilitates investigation of the upper digestive tract and collect tissue samples (biopsy) for further investigations [5]. Indeed, polymerase chain reaction (PCR) is a highly recommended assay to investigate the occurrence of bacterial infection and determination of bacterial replication in a variety of clinical biopsies and environmental samples [6]. Typically, *H. pylori* infects the body through the mouth, then transfers through the digestive system to infect the stomach or the first part of the small intestine. The flagellated bacterium can move around and burrow into the stomach lining, which stimulates the secretion of the pro-inflammatory cytokines [4]. Unlike other bacteria, *H. pylori* infection leads to overproduction of somatic acid in which it can survive by producing urease that neutralizes stomach acid. Furthermore, the produced ammonia has a harmful cytotoxic effect on human cells [4,7]. Since *H. pylori* has been classified as a class one carcinogenic pathogen and one of the critical risk factors for ulcer diseases and gastric cancer, it is likely that early diagnosis of *H. pylori* infection plays a protective role in cancer initiation and development [8]. Accordingly, raising attention has focused on the bacterial-host interaction and the alteration of cellular signaling associated with *H. pylori* infection [7]. Noteworthy, *H. pylori* can interact with more than forty host protein receptors via its type IV secretion systems (T4SS), such as cluster of differentiation 18 (CD18), CD46, CD74, E-cadherin, fibronectin, laminin, T-cell receptor, Toll-like receptors, and receptor tyrosine kinases EGFR. The bacterial T4SS includes serious proteins and effectors such as CagL, CagI, CagY, and CagA that can modulate cellular immune response to support bacterial vacuolation and intracellular replication [9,10]. In addition, *H. pylori* infection activates the intracellular receptors NOD1, NOD2, and NLRP3 with crucial impact in cellular immune response and pro-inflammatory cytokines secretion [11,12]. Further, *H. pylori* increases the DNA methylation of many genes in gastric epithelial cells suggesting the role of bacterial infection in the epigenetics alteration of the host genes that support cancer initiation [13]. Recent evidence indicates that *H. pylori* infection induces cellular autophagy via the bacterial effector VacA, however the endoplasmic reticulum (ER) stress and lysosomal inhibition by bacterial proteins stimulates the autophagy-induced cell death [14]. Cellular autophagy is the evolutionarily conserved machinery which depends on the lysosomal-catabolic degradation of cellular macromolecules and organelles engulfed by the autophagosomes and degraded by lysosomal-fusion [15–17]. The autophagic machinery is activated due to the exogenous environmental stress and the intracellular damages with the main purpose to maintain cell survival. The interruption of any stage of autophagy process including pre-autophagy, autophagosomes formation, and lysosomal-fusion leads to the initiation of the programmed cell death (PCD). In the current study, we sought to establish an accurate method to detect *H. pylori* in contaminated water and infected cells *in vitro* using the real time-PCR (RT-PCR) and specific oligonucleotides for 16SrRNA. Moreover, we tested the alteration of some host gene expression following *H. pylori* infection associated with cell proliferation and survival maintenance such as Raf-1, PI3K, and autophagy-related Atg5. Finally, we monitored the concentration of the pro-inflammatory cytokines produced upon *H. pylori* infection in HeLa cells including IL-1α, IL-1β, IL-6, and TNF-α.

2. Materials and Methods

2.1. Samples collection and genomic DNA isolation

The contaminated tap water samples were collected from different provinces in Egypt. To isolate the genomic DNA from the organic contents, the samples were centrifuged for 10 minutes at 5000 rpm using 15 ml falcon tubes, then the supernatant was removed. DNA lysis buffer was added to the pellet on each sample, then the genomic DNA purification kit (Thermo Fisher, USA) was used to isolate and purify the genomic DNA. The concentration of isolated DNA has been

measured by the NanoDrop and the final concentration has been adjusted to be 1 µg/µl.

2.2. Cell line

The cervical cancer cell line, HeLa cells (VACSERA, Egypt), was cultured in RPMI medium supplement with 4 mM l-glutamine and 10% fetal bovine serum (FBS) in a humidified 5% CO_2 atmosphere at 37°C. HeLa cells were seeded in 6-well tissue culture plates at a density of $2x10^5$ and were grown to 90% confluency. At two hours before infection, the medium was replaced by fresh medium containing 0.5% FBS, and the cells were allowed to grow for two hours pre-infection [18,19]. Finally, the bacteria were added to the cells for the indicated time points.

2.3. Bacterial strain and infection protocol

H. pylori strains P12 (wild type, USA) were cultured on agar plates containing 10% horse serum under microaerophilic conditions at 37°C and 10% CO_2 for five days. Then, the *Brucella* broth liquid medium supplemented with 10% (v/v) heat-inactivated FBS was used to culture and grow *H. pylori in a liquid media as previously described [20]. Different aliquots of the grown H. pylori in the liquid media have been stored at -80*°C. For the *in vitro* infection, the bacteria were harvested from culture plates in Dulbecco's phosphate-buffered saline (PBS) (pH 7.4) and the concentration of bacteria was measured using mass spectrophotometer by optical density at 600 nm, corresponding 1.0 is approximately equal 1×10^8 colony forming units (CFU)/mL [21]. The bacterial stock was added to the HeLa cells at different multiplicities of infection (MOI of 1, 5, and 10) for 6, 12, 24, 48, and 72h. As controls, PBS was incubated with the cells for the same time points. During infection, cells were monitored using an inverted microscope [22,23].

2.4. Cell viability and cytotoxic effects

Count and imaging of living cells upon infection have been used as indicators for cell viability rate at each time point. Assessing of lactate dehydrogenase (LDH) released into the media of infected cells has been used as an indicator of the potential cytotoxic effect of *H. pylori* infection on HeLa cells. The LDH assay kit (Ambion, USA) was used to assess the production level of LDH upon infection. According to the manufacture procedures, 10 µl of each sample was incubated with 40 µl LDH assay buffer for 10 minutes, then 2 µl LDH substrate was added, and LDH was measured immediately at OD 450nm in a microplate reader. The relative LDH production was calculated by dividing the mean absorbance values of the samples on the mock absorbance values which resulted in the fold change of LDH production [19,24].

2.5. Total RNA isolation and cDNA synthesis

Total RNAs were isolated from HeLa cells using TriZol (Invitrogen, USA) and chloroform and were purified by using RNA isolation kit (Invitrogen, USA). The purified total RNA was dissolved in RNase-free water and the concentration of all samples was adjusted to the final concentration of 100ng/ul. Then, the cDNA synthesis kit (Qiagen, USA) was used to generate the cDNA by using 15 µl from the purified RNA. According to the manufacturer protocol, total RNA was incubated with dNTPs, reverse transcriptase, and oligo (dT) primers at 45°C for one hour followed by 5 minutes at 95°C. The cDNA was then used for RT-PCR analysis and conventional PCR investigations.

2.6. Conventional PCR assay

The purified genomic DNA from contaminated water was used to amplify the 16SrRNA specific segment (250 nucleotides in length) using the specific primer sequences F: 5'-TCGGAATCA CTGGGCGTAA -3' and R: 5'-TTCTATGGTTAAGCCATAGGATTTCAC-3'. The following reagents have been prepared for each reaction; 15 µl PCR master mix, 100 ng of genomic DNA, 10 pmol from each premier (1 µl) and finally the total volume was adjusted to 25 µl with RNase- and DNase-free water. PCR was carried out in AmpGene DNA thermal cycler and the following PCR parameters were used: the initial denaturation step (94°C for 5 min), then 40 cycles (94°C for 30sec, 60°C for 20sec and 72°C for 30sec). Finally, the PCR product has been electrophoresed on 1% agarose gel for 30 min and monitored by gel documentation system [25].

2.7. Quantitative RT-PCR investigation

Quantitative RT-PCR (qRT-PCR) was used to detect the relative expression of *H. pylori*-16SrRNA,

Raf-1, Atg5, and PI3K genes to investigate both bacterial replication and cellular immune response in infected HeLa cells. The resulting cDNA was used as template for subsequent PCR amplification using primers specific for one gene. The relative gene expression of the indicated genes were detected using the QuantiTect SYBR Green PCR Kit (Qiagen, USA) and oligonucleotides specific for each individual gene, Raf-For-5´-TTTCCTGGATCATGTTCCCCT -3´, Raf-Rev-5´- ACTTTGGTGCTACAGTGCTCA -3´ [26], Atg5-1-For-5´-CGTGTATGAAAGAAGCTGATGC -3´, Atg5-Rev-5´- ACGAAATCCATTTTCTTCTGGA -3 [27], and PI3K-For-5´-TGCTGAACCCTATTGGTG -3´, PI3K-Rev-5´-TACAGTCCAGAAGCTCCA -3 [28]. Levels of GAPDH were amplified using specific oligonucleotides, GAPDH-For-5'-TGGCATTGTGGAAGGGCTCA-3' and GAPDH-Rev-5'-TGGATGCAGGGATGATGTTCT-3' [16], which was used for normalization as internal control. The following parameters have been used in RT-PCR program, 94°C for 5 min, 40 cycles (94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds) and held at 4°C. The indicated Ct values have been analyzed using ΔΔCt equations [29,30].

2.8. Enzyme-linked immunosorbent assay

For quantitative measurement of secreted interleukins, sandwich enzyme-linked immunosorbent assay (ELISA) was used to determine the concentrations of IL-1β, IL-1α, IL-6 and TNF-α in the fluid media of infected cells. The human ELISA kits (Abcam, 46052), (Abcam, 46028), (Abcam, 46042), and (Abcam, 181421) have been used to detect the indicated cytokines, respectively. Accordingly, HeLa cells were seeded and incubated overnight in 96-well plates, at a density of $2x10^4$ cells per well. The cells were infected with *H. Pylori*, MOI of 10 and were incubated for different time points (0, 6, 12, 24, 48, and 72 hours). The infected cells were then lysed and transferred into the ELISA-96-well plate followed incubation at 37°C for 4 hours. The media was then removed, and the cells were washed using PBS, then 100 µl of the antibody solution was added to each well followed by one-hour incubation at RT. Then, 200 µl of the substrate was added to each well followed by 15 minutes incubation away from the light at RT. Finally, the reaction was stopped by adding 50 µl from stop solution and the intensity of the color was measured at 450 nm [31–33].

2.9. Agarose gel electrophoresis

The conventional PCR products were loaded in 1% agarose gel which has been prepared by dissolving 1 g agarose in 50 ml TAE (1X) in a graduated cylinder and then was transferred into the agarose flask. The top of the flask was covered with a paper towel and was boiled at microwave until completely milt. 10 µl of the ethidium bromide was added to the warm gel which then was poured to the DNA electrophoresis cassette and left to be cold. After loading the samples onto the gel, the electrophoresis was carried out using 50 V for one hour [25].

2.10. Statistical analysis

The qRT-PCT data analysis was performed by driving the ΔΔCt values using the following equations; (Delta Ct) = Ct-gene – Ct-GAPDH. (ΔΔCt) = ΔCt-sample – ΔCt- control. Finally, the relative gene expression (indicated by fold change) is equal (2-ΔΔct) [29,34].

3. Results and Discussion

3.1. *H. pylori* infection induces cytotoxic alteration in HeLa cells

To investigate the potential cytotoxic effect of *H. pylori* in infected HeLa cells, the number of living cells and representative cell image have monitored in infected cells. Additionally, LDH production from infected cells has been measured to investigate the possible cytotoxic effects following infection. Therefore, HeLa cells were seeded in 6-well plate in concentration of 2×10^5 cells per well followed by overnight incubation at CO_2 incubator. HeLa cells were infected with different MOI of *H. pylori* (1, 5, 10 MOI) followed by 2 days incubation. Expectedly, *H. pylori* infection showed an obvious cytotoxic effect in HeLa indicated by apoptotic cell images and decreased number of living cells, approximately 0.5 fold change in day 1 and 2.5 fold change in day 2 (Figure 1A and B). Further, LDH production was measured as an indicator for cytotoxic alteration in

infected and non-infected cells. Noteworthy, LDH is an enzyme that converts lactic acid to pyruvic acid in Krebs's cycle. Importantly, LDH is found in mitochondria of all living and its secretion has toxic effect that stimulates PCD. Here, the level of LDH production was increased up to 3 fold changes in day 1 following infection and 4 fold changes in day 2 that further confirms the cytotoxic influence of *H. pylori* infection on cell proliferation (Figure 1C). These findings indicate that *H. pylori* infection stimulates PCD in infected HeLa cells.

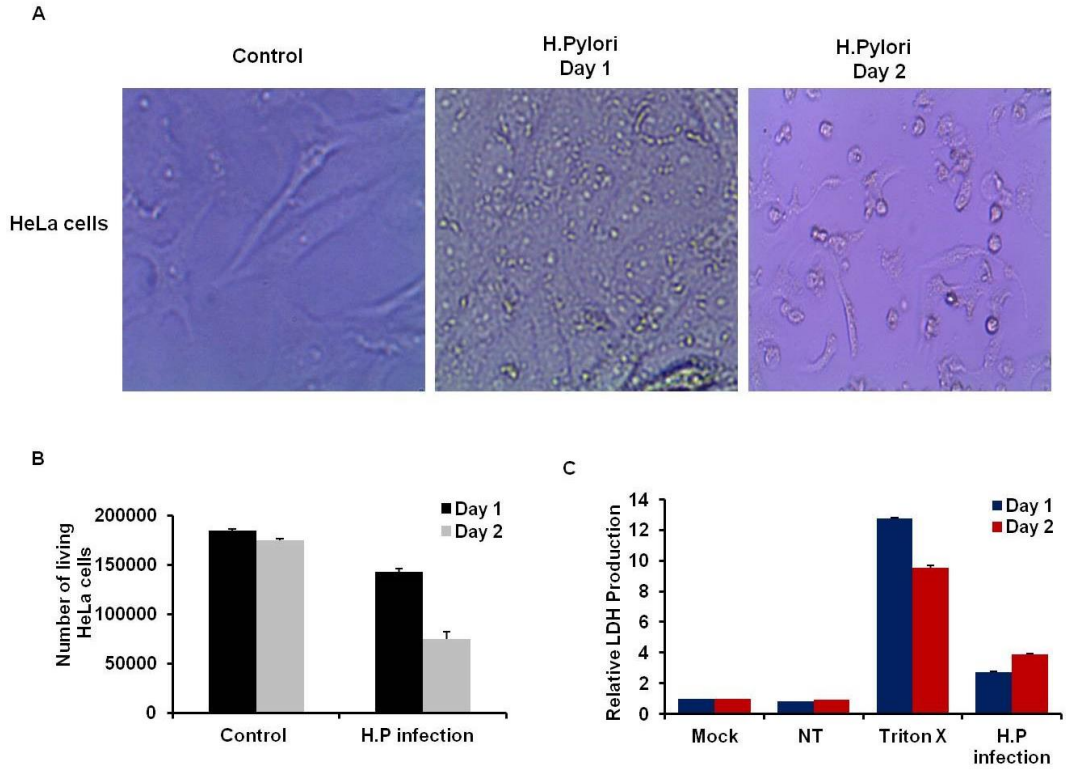

Figure 1. Cytotoxic effect of *H. pylori* infection in HeLa cells.(A) The representative images of Hela cells that were infected with H. pylori in day 1 and day 2 compared with non-infected by using inverted microscope (40x). (B) number of living cells upon infection that were manually calculated (C) relative LDH production from infected cells compared to Triton X-100 and non-infected cells (NT). Error bars indicated the standard deviation (SD) of two independent experiments.

3.2. Detection of *H. pylori* using reverse transcriptase and RT-PCR

Based on the differentiation of 16SrRNA in different pathogens, we designed new specific oligonucleotides for *H. pylori-16SrRNA* to figure out bacterial replication in infected cells using RT-PCR. Meanwhile, by using the same created primer, the expected amplified fragment from *H. pylori*-genomic DNA is almost 300 nucleotides in length using reverse transcriptase PCR. Total RNA was first isolated and purified from infected HeLa cells and has been loaded in agarose gel to verify the purification of both 16SrRNA and 18SrRNA (Figure 2A). For RT-PCR analysis, total RNA has been isolated and purified from HeLa cells that were infected for 2 days with the indicated MOI of the *H. pylori*. The relative gene expression of 16SrRNA was detected in infected cells and was normalized to the infected cells with MO1=1 (1X). The results revealed that the relative expression of *H. pylori* -16SrRNA was gradually increased in a dose-dependent manner of *H. pylori* infection when compared with the lower infection (Figure 2B). Furthermore, by using purified DNA, the expression of *H. Pylori*-16SrRNA in contaminated water has been successfully recognized in the agarose gel using conventional PCR when compared with the standard strain of *H. pylori* (Figure 2C). These data indicate that our established method using specific primer for *H. pylori*-16SrRNA is sufficient to detect and confirm the presence and

replication of *H. pylori* in infected cells and contaminated water samples, respectively.

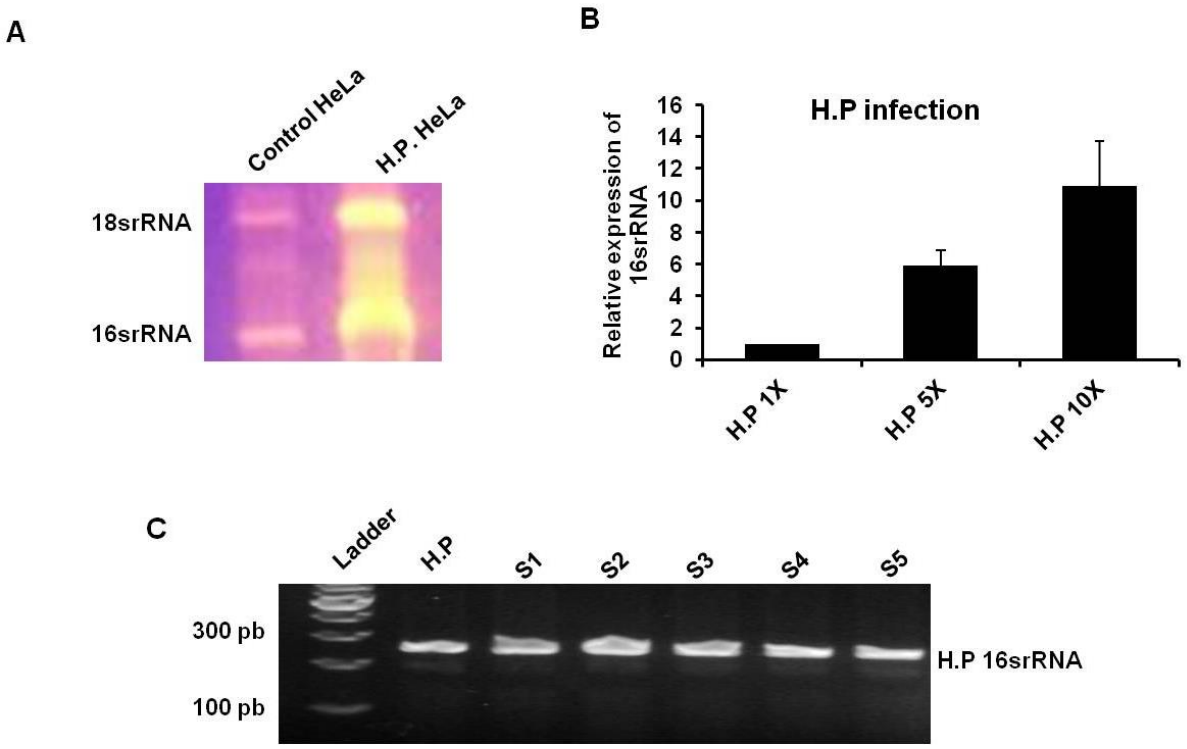

Figure 2. Recognition of *H. pylori* in infected HeLa cells and contaminated water. (A) Agarose gel electrophoresis indicates total RNA that isolated and purified from infected and non-infected HeLa cells. (B) Fold changes in *H. pylori*-16SrRNA expression in HeLa cells that were infected with different dose of *H. pylori* (MOI=1, MOI=5, and MOI=10) using RT-PCR. Error bars indicate the standard deviation (SD) of two independent experiments (C) Agarose gel electrophoresis reveals the molecular size of amplified fragment by using *H. pylori* genomic DNA and premiers specific for 16SrRNA.

3.3. *H. pylori* infection stimulates the expression of cell proliferation genes

HeLa cells were seeded in 6-well plate with a density of $2x10^5$cells/well and were infected with *H. pylori*, MOI of 10 for 2 days. The relative gene expression of Raf-1 and Atg5, and PI3K in infected HeLa cells was quantified by using q-RT-PCR and normalized to non-infected cells. Interestingly, the relative expression of Raf-1 was significantly increased (up to 5-fold) in infected HeLa cells in comparison with the control-cells (Figure 3A). Likewise, the relative expression of the autophagy-related Atg5 was increased up to 8 fold changes in infected cells compared with non-infected cells (Figure 3B). Furthermore, the steady state mRNA of PI3K was increased more than 2 fold changes as a response to *H. pylori* infection when compared with non-infected cells (Figure 3C). Together, these data suggest the increasing levels of the steady state mRNA of Raf-1, autophagy-related Atg5, and PI3K as a response to *H. pylori* infection in HeLa cells.

3.4. *H. pylori* infection increases production of IL-6 and TNF-α from infected HeLa

To achieve the time-dependent competence of the *H. pylori* infection on the secretion of the pro-inflammatory cytokines, HeLa cells were seeded in 96-well plate and were incubated for different time points at 6, 12, 24, 48, and 72 hours following infection. Then, the pro-inflammatory cytokine secretion, including levels of produced IL-1α, IL-1β, IL-6, and TNF-α was monitored in the fluid media of infected cells. The results showed that the concentration of both IL-1α and IL-1β were comparable in both infected and non-infected cells during all different time points (Figure 4A and B). In contradictory with this, the concentration of produced IL-6 and TNF-α were

increased in a time-dependent manner following infection (Figure 4C and D). Collectively, these findings indicate that *H. pylori* infection regulates the production of the pro-inflammatory cytokines in a time-dependent manner to overcome the cellular immune response of neighboring non-infected cells.

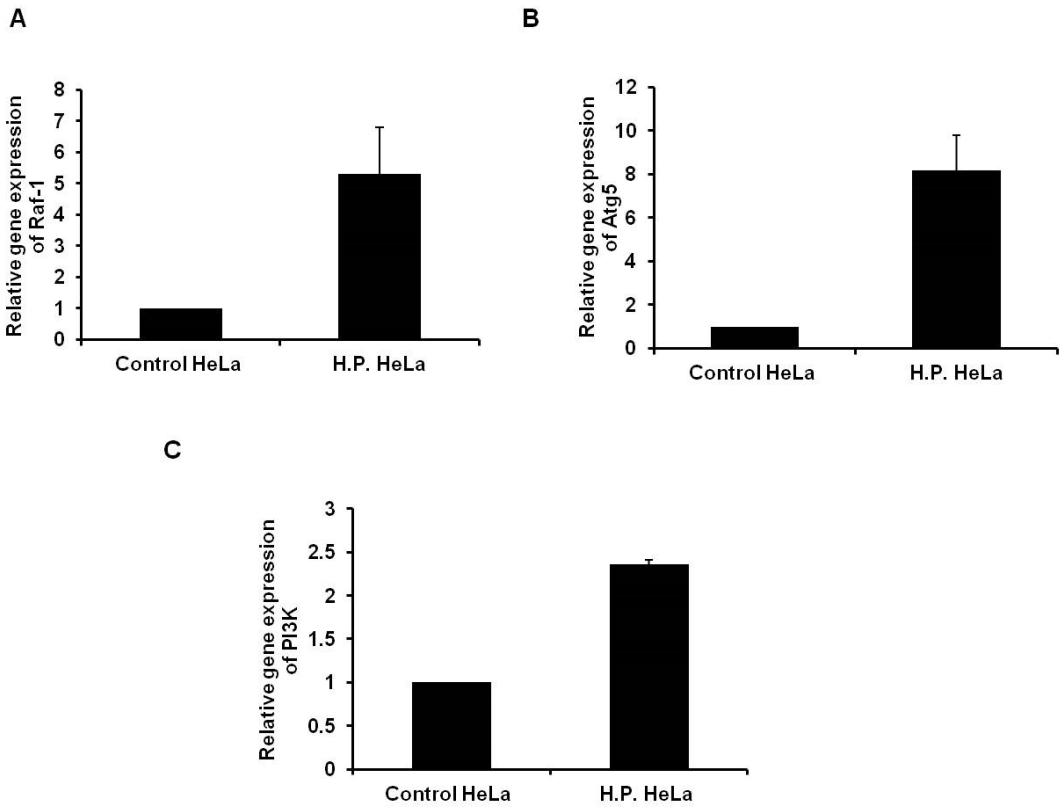

Figure 3. Relative expression of the host factors associated with *H. pylori* infection in HeLa cells. (A) Relative gene expression of Raf-1 in infected HeLa cells indicated by fold changes and normalized to non-infected cells (control HeLa). (B) Fold changes of the autophagy-related Atg5 expression in infected cells in comparison with non-infected cells. (C) Fold changes of the PI3k gene expression upon *H. pylori* infection in HeLa cells compared to control cells. Error bars indicate the SD of two independent.

In the current work, we successfully established an accurate method using both the conventional and RT-PCR.to detect and recognize *H. pylori* in contaminated water and infected HeLa cells by specific oligonucleotides for the *H. pylori* 16SrRNA.Furthermore, we investigated the relative gene expression and cytokines secretion associated with *H. pylori* infection *in vitro*. Interestingly, our findings confirm the possible identification of *H. pylori* in contaminated water using the purified genomic DNA which revealed a specific fragment with a molecular size of about 250 base pairs, when amplified with the primers specific for *H. pylori*-16SrRNA. Moreover, the relative expression of cell proliferation and cell survival genes including Raf-1, Atg5, and PI3K was markedly increased upon *H. pylori* infection in HeLa cells suggesting the possible impact of *H. pylori* in maintaining cell survival following infection to avoid the cytotoxic effects of bacterial vacuolation. Meanwhile, the cytotoxic effects of *H. pylori* infection have been monitored dependent on the levels of secreted LDH from infected cells. The present data reveal that LDH production is increased in time-dependent following *H. pylori* infection as a result of mitochondrial stress and oxidative stress (hypoxia). *H. pylori*-induced mitochondrial stress has been recently reported through the regulatory function of its VacA effector which increases mitochondrial dysfunctional and hypoxia [35]. Noteworthy, the bacterial-induced oxidative stress is one of the recognized mechanism by which *H. pylori* activates apoptotic signaling in the late stage of infection [36]. LDH is one of the hypoxia

biochemical parameters that reveal the oxidative stress condition by increasing levels of its extracellular activity. LDH is an enzyme located in mitochondria in all living cells responsible for the conversion of lactate to pyruvic acid during the Krebs cycle. Importantly, the secretion of LDH refers to a systemic toxic effect on cell proliferation that induces PCD [31,37,38]. The patients with chronic infection of *H. pylori* showed high production levels of the pro-inflammatory cytokines IL-17 and IL-23 [39]. The children infected with *H. pylori* showed increasing levels of the pro-inflammatory cytokines IL-6, IL-10, IFN-γ, TNF-α, at both RNA and protein levels [40]. Similarly, long-term infection with *H. pylori* triggers the pro-inflammatory cytokines IL-1β, as a key mediator of many pathogen-physiological events and reveals host-environment interactions [41]. Likewise, our findings further confirm that the cytotoxic effects of *H. pylori* infection may be started in day 2 following infection indicated by the high production levels of LDH and the pro-inflammatory cytokines IL-6 and TNF-α. The increased production levels of both IL-1α and IL-β were not detectable until day 3 following infection, indicating the sequence of the cytokines secretion events from infected cells. Conversely, in the early stage of infection, *H. pylori* activates *in vitro* cell proliferation signals including the mitogen-activated protein kinases (MAPK), nuclear factor-κB (NF-κB), and the activator protein 1 (AP-)1 in a dose-dependent manner [42,43]. The immediate activation of the cell survival pathway PI3K/AKT signaling has been reported following *H. pylori* infection in maintaining gastric cancer cells [44].

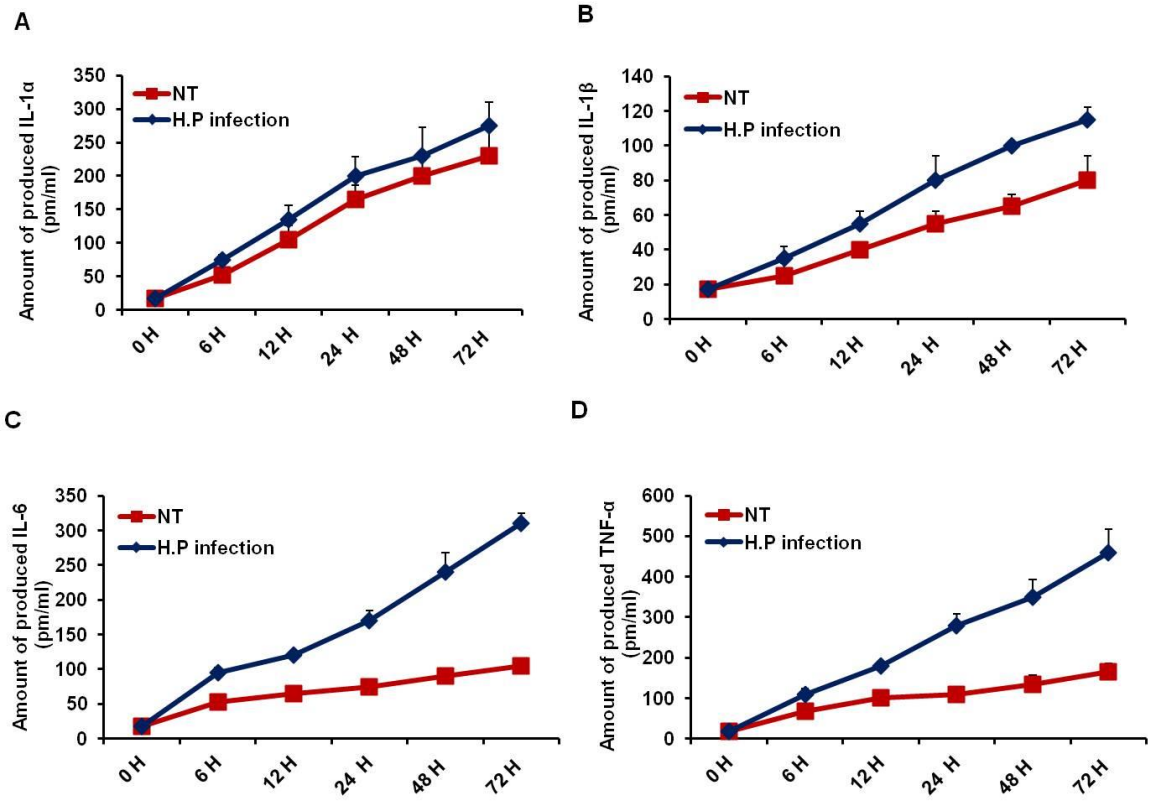

Figure 4. Levels of pro-inflammatory cytokines produced from infected HeLa cells. (A and B) The concentration of IL-1α and IL-1β (pm/ml) produced from infected HeLa cells at different time points in comparison with non-infected cells (NT). (C and D) Level of IL-6 and TNF-α (pm/ml) produced from infected HeLa for the indicated time points compared with NT. Error bars indicate the SD of 4 different replicates.

Furthermore, the bacterial effector, VacA has been identified as an efficacious regulator of the mammalian target of rapamycin complex 1 (mTORC1) which resulted in activation of autophagy and preventing PCD [45]. Likely, we demonstrated the over-expression of some regulator genes in infected HeLa cells including the expression of Raf-1, as an indicator for

MAPK signal, Atg5, as an indicator for autophagy process, and PI3K as an indicator for the cell survival pathway PI3K/AKT. Therefore, the present data coincide with the previous findings indicating that *H. pylori* infection activates the cell survival signaling following infection to support the intracellular replication and to avoid the apoptotic signaling with a minimum cytotoxic response. In the late stage of infection *H. pylori* stimulates the apoptotic signaling and triggers the production of the pro-inflammatory cytokines to overcome the cellular immune response of the neighboring cells.

4. Conclusions

We established an accurate and sensitive method to detect *H. pylori* in the contaminated water via isolation of the genomic DNA from collected samples and amplification of the purified DNA with specific primers for the *H. pylori*-16SrRNA. Intracellular replication of *H. pylori* has been also successfully monitored using the same oligonucleotides in infected HeLa cells. The investigation of cellular signaling associated with *H. pylori* infection further confirms the ability of *H. pylori* to maintain cell survival and to avoid apoptotic signaling following infection. Furthermore, the cytotoxic effect of the *H. pylori* infection was detected at day 2 of the infection indicated by an increased level of LDH, IL-6, and TNF-α *in vitro*.

Acknowledgments

The authors declare no acknowledgments.

Conflicts of Interest

The authors declare no conflict of interest.

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