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IMPACT OF SUB-INHIBITORY CONCENTRATIONS OF AMOXICILLIN ON *HELICOBACTER PYLORI* VIRULENCE FACTORS

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ABSTRACT

Helicobacter pylori are etiological factor of human acute and chronic gastritis. Critical host responses that influence the progression to *H. pylori*-induced carcinogenesis include gastric inflammation. This study was conducted to evaluate whether subinhibitory concentrations of amoxicillin can affect *H. pylori*-induced cytokines by RAW 264.7 macrophage-like cells. In addition, a proteomic approach was used to compare *H. pylori* protein expression profiles under amoxicillin treatment. *H. pylori* ATCC 43504 was cultured with or without amoxicillin at subinhibitory concentrations (sub-MICs). The cytokines (IL 1-β, IL-6, IL-12, and TNF-α) released by infected RAW 264.7 cells were quantified by a sandwich immunoassay. Differential protein analysis of *H. pylori* was performed using 2-dimensional electrophoresis (2-DE) followed by mass spectrometry and the sequences were searched against the SWISS-PROT data bank. Pre-treatment H. *pylori* with sub-MIC of amoxicillin increased IL-1β and IL-12 production by RAW 264.7 cells. Proteomics analyses of proteins revealed that the sub-MIC treatment affected the expression of several classes of proteins. Among the nine most over expressed proteins included heat shock proteins, superoxide dismutase, enolase, ATP synthase alpha subunit, neutrophil activating protein and argininosuccinate synthase. The results shown in the present work indicate that sub-MICs of amoxicillin can induce specific changes on the

cell wall proteins and behavior of susceptible *H. pylori* that can contribute to the pathogenesis of the bacterium.

KEY WORDS: *Helicobacter pylori*, amoxicillin, sub-inhibitory concentration, cytokines, macrophage, proteomics.

INTRODUCTION

Helicobacter pylori is a Gram-negative bacterial species that selectively colonizes gastric epithelium and is the most common bacterial infection worldwide.[1] Among many unique characteristics of *H. pylori*, one of the most remarkable is its capacity to persist for decades in the harsh gastric environment due to an inability of the host to eliminate the infection.[2] Several studies have now provided clear evidence that *H. pylori* infection significantly increases gastric cancer risk.[3][4] Critical host responses that influence the progression to *H. pylori*-induced carcinogenesis include gastric inflammation and a reduction in acid secretion.[1] Eradication of *H. pylori* significantly decreases the risk of gastric cancer in infected individuals without premalignant lesions. [2]

H. pylori adhesion to gastric epithelial cells constitutes a key step in the establishment of a successful infection of the gastric mucosa and it involves specialized molecular interactions that may lead to intimate attachment.[5] To colonize the human stomach for extended periods of time, *H. pylori* must not only overcome the physical and cellular barriers, but also avoid the innate and adaptive immune responses that are triggered in the stomach by its presence.[6] Infiltration of the gastric mucosa with neutrophils, macrophages, and lymphocytes is a marked feature of *H. pylori* infection. [6] This mechanism occurs predominantly on T helper type 1 cells that induce the production higher levels of cytokines and chemokines, such as interleukins (IL) IL-4, IL -1β, IL-6, IL-8, IL-10, IL-12, and tumour necrosis factor alpha (TNF-α), contributing to the inflammatory process, with consequent damage to mucosal cells.[7] The presence of *H. pylori* in the stomach leads to diminishment of the mucous protective layer due to the inhibition of mucus production by epithelial cells. [1]

Many different therapeutic regimens for *H. pylori* eradication have been used previously, but at present the most widely recommended treatment in international guidelines is the Standard Triple Therapy based on a proton-pump-inhibitor to which two antibiotics (clarithromycin plus amoxicillin or metronidazole) are associated for 7 days. [8] The efficacy of this therapy depends on antibiotic susceptibility pattern, and patient compliance. [3] Noncompliance

however, is a frequent problem in the outpatient setting. [9] Several studies have demonstrated that subinhibitory concentrations (sub-MICs) of antibiotics might alter the host-pathogen interaction. [10] [11]

The proteome is the entire complement of proteins that can help in understanding the pathogenic mechanism of organisms and provides a basis of the improvement of diagnosis and therapy. [12] It has been frequently demonstrated that proteomic analysis is a powerful approach used to characterize proteins produced by cells, tissues or organisms at a given time under specified conditions. [13]

To investigate whether amoxicillin can influence *H. pylori* infection, in this study we evaluated if subinhibitory concentrations (sub-MICS) can affect *H. pylori*-induced cytokines by RAW 264.7 macrophage-like cells. A proteomic approach was used to characterize and compare *H. pylori* protein expression profiles under amoxicillin treatment. Considering that amoxicillin is included in treatment regimens and given the enormous heterogeneity of *H. pylori* strains, the data can be use for additional investigation concerning to low-dose therapy that can affects the clinical outcomes.

MATERIALS AND METHODS

Helicobacter pylori strain, amoxicillin susceptibility and growth conditions

H. pylori ATCC 43504, metronidazole resistant and amoxicillin susceptible, was cultured in Columbia agar containing 10% fetal bovine serum and incubated at 37°C for 72 hours, in 5% CO_2. The minimal inhibitory concentration (MIC) of amoxicillin (Lot number: 080616478, Pharma Nostra, Rio de Janeiro, Brazil) was determined using the broth microdilution method as previously described [14]. The wells of a 96-well microplate were filled with 100 μL of various concentrations of amoxicillin. Same volume of *H. pylori* suspension (about 10^6 CFU/mL) was added to each well. The absorbance was determined using an automatic ELISA microplate reader (Spectra & Rainbow Readers, Tecan) adjusted at 620 nm. The microplate was incubated at 36–37°C for 3 days, under microaerophilic atmosphere, agitated and the absorbance was read again in the reader at the same wavelength. The absorbencies were compared to the values obtained before incubation to detect an increase in bacterial growth. The lowest concentration of the test amoxicillin resulting in inhibition of bacterial growth and the obtained MIC value was 0.25 μg/mL. *H. pylori* was cultured in Brain Heart Infusion supplemented with 10% fetal bovine serum in the presence (HPAMX) or absence

(HP) of amoxicillin at subinhibitory concentrations (sub-MICs) (½ and ¼ MIC) at 37ºC under microaerophilic atmosphere for 24 hours. In the cytokines assays, bacterial cells were washed twice with sterile phosphate buffered saline (0.05 mol/L and pH 7.2), centrifuged at 6000×*g* for 10 min, and suspended in Dulbecco's modified Eagle's medium in standard inoculum corresponding to 10^9 CFU/mL (UDO620 0.40). In the proteomic approach, bacterial cells were washed twice in icecold sterile phosphate buffered saline (0.05 mol/L and pH 7.2) containing protease inhibitors (1 mmol/L PMSF, Roche Applied Science) and centrifuged at 6000×*g* for 10 min. Bacterial cells were lyses in ice-cold sterile distilled water in the final concentration of 10^9 CFU/mL (UDO620 0.40) by sonication (20% amplitude, 6 pulses of 1 min with 30 seconds rest on ice). The lysate was centrifuged at 6000×*g* for 10 min. The supernatants were separated, aliquoted, and stored at −80°C. The protein concentration of the extracts was quantified by the Bradford method. [15]

RAW 264.7 macrophage-like cells stimulation and cytokine assays

RAW 264.7 macrophage-like cells (American Type Culture Collection ATCC, TIB 71, Rockville, MD) were cultured in Dulbecco's modified Eagle's medium containing L - glutamine and glucose (Gibco BRL Life Technologies) supplemented with 10% FBS and 10 μg/mL gentamicin (Gibco BRL Life Technologies) in air with CO_2 5% at 37°C. The cells (1 × 10^6 cells/500 μL/well) and HP or HPAMX (500 μL as described) were incubated for 24 hours at the same conditions. Each experiment was accompanied by controls (10 μg/mL lipopolysaccharide (Sigma), and Dulbecco's modified Eagle's medium). After incubation, the supernatants were collected and centrifuged (10 000×*g*, 5 min) to remove cells. The cytokines (IL-1β, IL-6, IL-12, and TNF-α) were quantified by a sandwich immunoassay kit (BD Biosciences Pharmingen) following the manufacturer's instructions. Briefly, 96-well plates were coated overnight with 100 μL/well of a purified rat anti-mouse cytokine capture antibody diluted in sodium bicarbonate buffer (pH 9.5), or sodium phosphate buffer (pH 6.5), and incubated overnight at 4ºC. The plates were washed three times with phosphate buffered saline (PBS 0.05 mol/L and pH 7.2) containing 0.05% Tween-20 (PBS-T). The plates were blocked with 300 μL/well of 10% fetal bovine serum (FBS) in PBS at room temperature for 1 hour. Plates were washed three times with PBS-T. Aliquots of 100 μL of samples (supernatant of macrophage cell culture) or standard murine cytokines were added to the appropriate wells. The plates were incubated at room temperature for 2 hours, washed five times with PBS-T, and 100 μL biotinylated goat antimouse cytokine detection monoclonal antibody plus streptavidin–horseradish peroxidase reagent, diluted in assay diluent (PBS +

10% FBS), was added to each well. The plates were incubated at room temperature for 1 hour, washed seven times with PBS-T and 100 µL of substrate solution (BD Pharmingen, San Jose, CA, TMB Substrate Reagent) was added to each well, the plates being incubated at room temperature for 30 min in the dark. The reaction was stopped by adding 50 µL Stop Solution (1 M H_2SO_4) to each well. Absorbance was read at 450 nm, within 30 min of stopping the reaction, on a microplate reader (Multiskan Ascent, Labsystems) and cytokine concentrations were calculated from a curve of known concentrations of each cytokine standard. The results were expressed in pg/mL. Experiments were carried out in triplicate and repeated at least twice. The results were expressed through mean ± sd. Statistical comparisons were performed using Student's t test data ($p < 0.05$).

Two-dimensional electrophoresis (2-DE) protein separation

Preliminary evaluation of the extracts was performed using SDS–PAGE with a 12.5% acrylamide gel. Ten micrograms (10 µg) of each extract were applied to the gel, and the proteins were separated at a constant 200 V. Protein bands were revealed by colloidal Coomassie Blue (G-250, Biorad, Hercules, CA) staining. Two-dimensional electrophoresis (2-DE) sample preparation was carried out as described previously.[16] Protein concentration was determined by the Bradford method.[15] The solubilized protein samples (100 µg) were mixed with the DeStreak rehydration solution (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), to a final volume of 320 µL and applied to narrow range IPG gel strips (pH 3–10, 13 cm, Amersham Pharmacia Biotech). After the IPG strips were subjected to passive rehydration overnight at room temperature and focusing was performed with the Ettan IPGphor system (GE Healthcare) at 20°C using the following program: first conditioning step (15 min at 250 V), the linear voltage ramping step (3 h at 10 kV), and the maximum voltage ramping step of up to 90 kV. After the run, the strips were incubated for 15 min in equilibration buffer (50 mmol/L Tris–HCl, pH 8.8, containing 6 mol/L urea, 30% glycerol, 2% SDS and 0.02% bromophenol blue) that contained 1% DTT for reduction. The proteins in the strips were alkylated in equilibration buffer containing 2.5% iodoacetamide for 15 min. The proteins were resolved in the second-dimension in 12.5% polyacrylamide gels using the Ettan Dalt Twelve (GE Healthcare) at 1.5 W per gel. Preparative gel has been obtained, through the above described procedure, but with a 1000 µg total protein load. After 2-DE the gel was fixed in 50% ethanol and 2% ortophosphoric acid followed by an exposure to a staining solution (17% $(NH_4)_2SO_4$, 2% ortophosphoric acid, 34% methanol). Coomassie Colloidal G-250 was added to a final concentration of 0.065%. Destaining of the gel was

performed with ultrapure water until the background was completely clear. Coomassie-stained gel was scanned with an ImageScanner (Amersham Biosciences).

Image acquisition and analysis

The individual gels were imaged by using an ImageScanner (Amersham Biosciences) with an 8-bit grey scale 400 dpi transparency adapter. Spot detection, quantification (% volume), and pattern matching were performed by using the ImageMaster TM 2D Platinum software version 5.0 (Amersham Biosciences). The total density of each gel image was used to normalize the individual spot volumes and minimize inter-gel variations. Each spot volume was normalized as a percentage of the total volume of all the spots present in a gel. The amount of protein in each spot was expressed in terms of % volumes. Gel-to-gel comparisons between proteins profile from experiments at 0.063 µg/mL (¼ MIC), 0.125 µg/mL (½ MIC), and control after 24 hours incubation were performed for three independently prepared samples. We set a high stringency parameter of 2- and 3-fold differences in the percentage volume as the thresholds for identifying the differentially expressed proteins. The spots identified as differentially expressed in the triplicate comparisons were subjected to mass spectrometry (MS) analysis.

Protein digestions and MS analysis

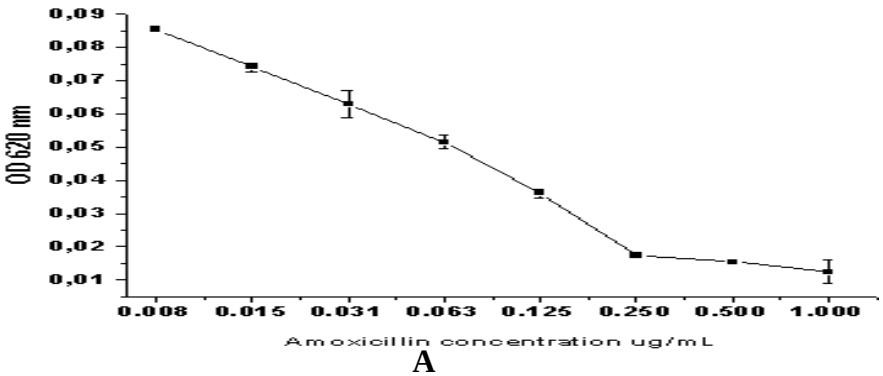
Spots were excised from gels and destained in 50% acetonitrile (ACN) and 25 mmol/L ammonium bicarbonate. Gel plugs were dehydrated with 100% ACN and allowed to air dry after removal from solvent. The gel pieces were rehydrated with 20 ng/mL trypsin solution (Sigma) in 50 mmol/L ammonium bicarbonate, and the tubes were incubated for 12 h at 37°C. To extract the peptides, the gel pieces were incubated twice with 50 µL of 50% v/v ACN and 5% v/v formic acid (FA), and once with 50 µL of ACN. All supernatants were combined and vacuum dried, and the pellets containing the peptides were resuspended in 0.1% v/v FA for MS analysis. For LC-MS/MS, the digested samples were injected using the nanoAcquity UPLC sample manager and the chromatographic separation was performed using a UPLC C18 column (75 µm× 10 cm) with a flow of 1 µL/min. The mass spectra were acquired in a Synapt HDMS instrument (Waters Co., Milford, MA, USA) using a data dependent acquisition (DDA), where the three top peaks were subjected to MS/MS. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The gradient conditions used were as follows: 0 min with 3% of B, increasing linearly to 30% B in 20 min, then it increased up to 70% B in 40 min where it remained until 50 min and in the next minute it was decreased to 3% of B. The data were processed using

Mascot Distiller (Matrix Science Ltd) and subjected to database search using Mascot Server 2.3. The searches were made with the assumption that there was a maximum of one missed trypsin cleavage and that peptides were mono-isotopic and using partially oxidized methionine residues, and completely carbamidomethylated cysteine residues. Peptide mass tolerance and fragment mass tolerance were initially set to ±0.1 Da, respectively, for MS/MS ion searching. However, candidate peptide IDs were only accepted if the m/z values were observed within 0.1 Da (typically less than 0.05 Da) of the theoretical mass of the candidate ID. Protein identification has been performed using the MASCOT search engine http://www.matrixscience.com and Pro-Found http://prowl.rockefeller.edu/prowl-cgi/profound.exe peptide mass fingerprinting (PMF) tools. The NCBInr and Swiss Prot were used as the protein sequence databases, to produce a standardized probabilistic measure of confidence.

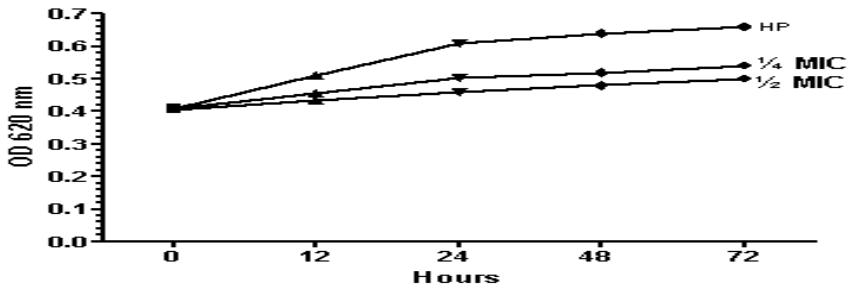
RESULTS

Effect of amoxicillin on *Helicobater pylori*

The MIC of amoxicillin for *H. pylori* ATCC 43504 and the growth curves in the presence or absence of amoxicillin at sub-MICs (½ and ¼ MIC) for 24 hours is shown in Fig. 1.



A



B

Fig. 1 Effect of amoxicillin on growth of *Helicobacter pylori.* (A): Minimum inhibitory concentration; (B): Grown curve of *Helicobacter pylori*: HP: untreated *H. pylori*; ¼ MIC: *H. pylori* cultured with amoxicillin 0.063 μg/mL; ½ MIC: *H. pylori* cultured with amoxicillin at 0.125 μg/mL.

Effect of *Helicobacter pylori*-induced cytokines by RAW 264.7 macrophage-like cells

The findings presented in Fig. 2 shows that treatment of HP with 0.063 µg/mL (¼ MIC) and 0.125 µg/mL (½ MIC) of amoxicillin increased IL-1β and IL-12 ($p<0.05$) from RAW 264.7 infected cells but did not altered TNF-α and IL-6 compared to that induced by HP not exposed to antimicrobial.

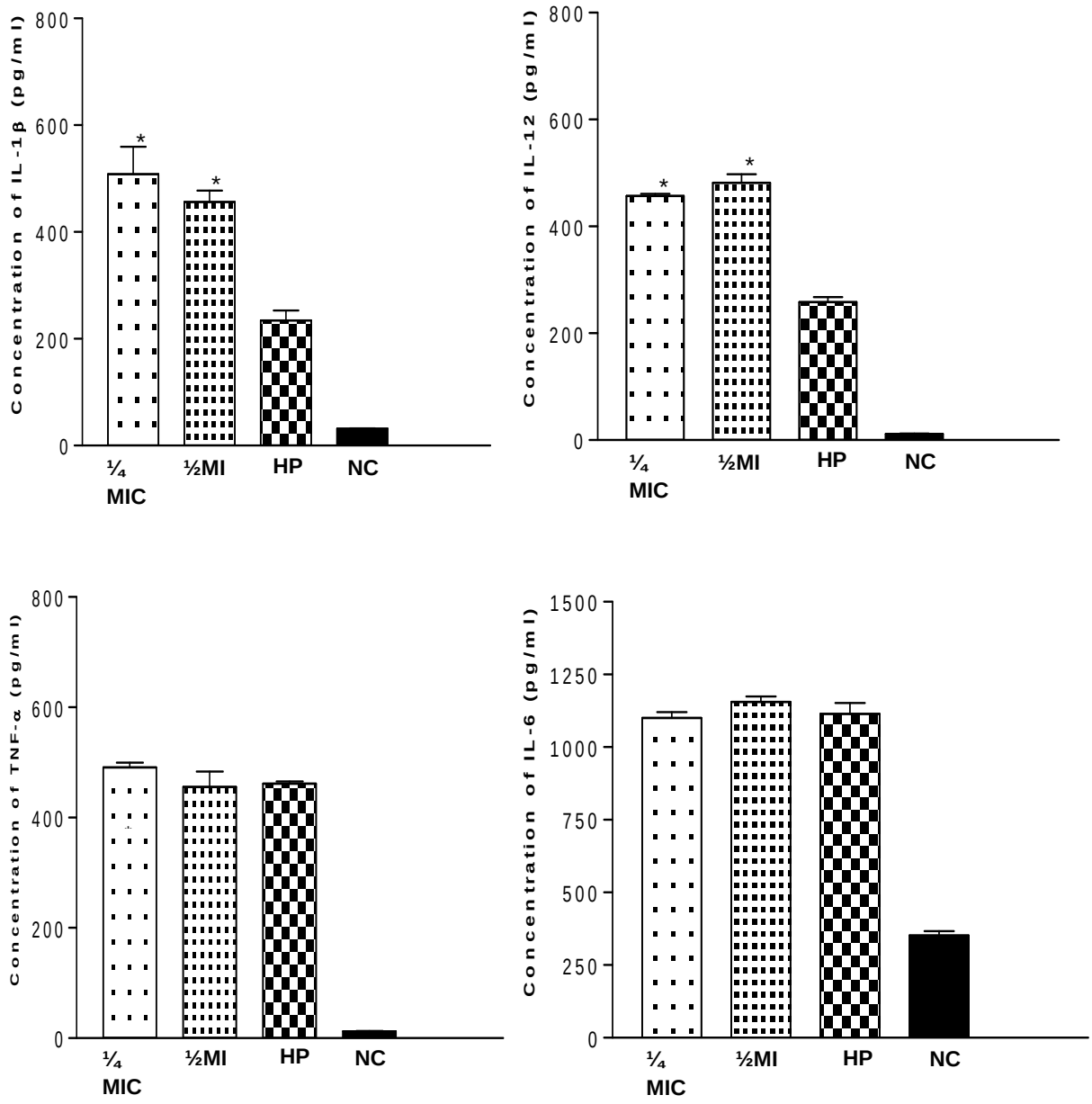
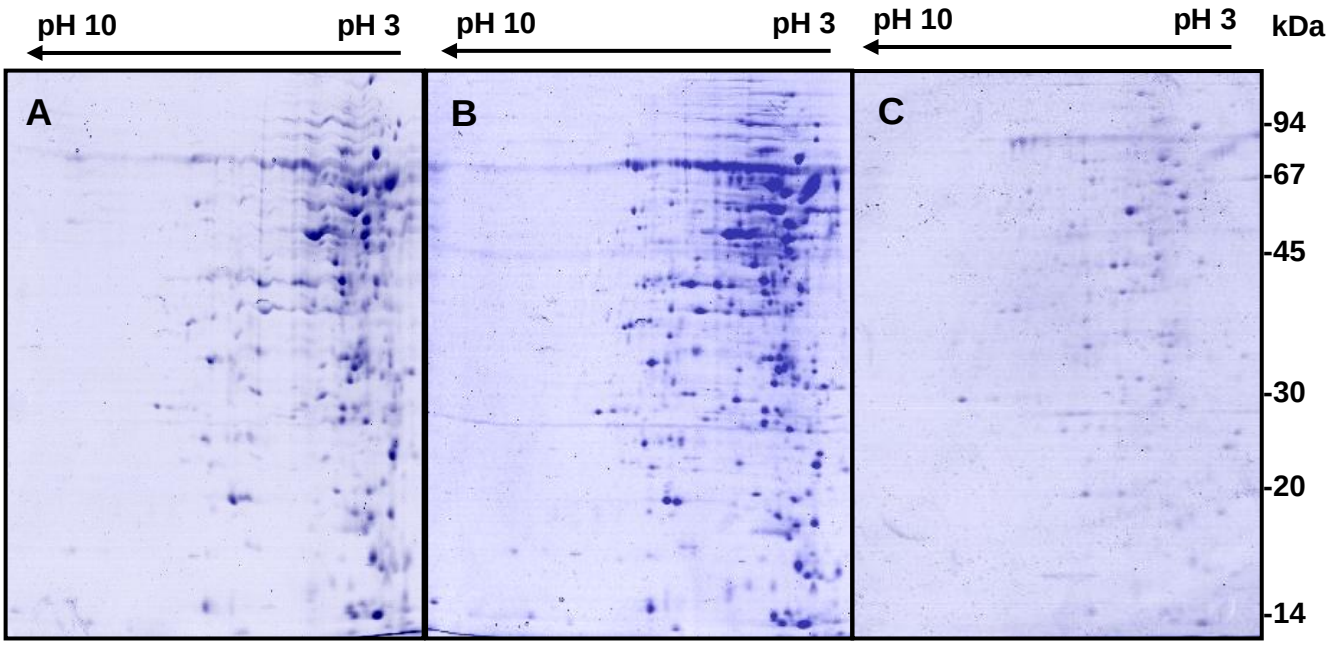


Fig. 2 Cytokines released by RAW 264.7 cells treated with *Helicobacter pylori* cultured with amoxicillin at subinhibitory concentrations for 24 hours. A total of 1×10^6 cells/500 µL/well were infected with *H. pylori* (10^9 CFU/mL). Concentrations of cytokines in the supernatants were determined by a sandwich immunoassay. ¼ MIC: *H. pylori* cultured with amoxicillin at 0.063 µg/mL; ½ MIC: *H. pylori* cultured with amoxicillin at 0.125 µg/mL; HP: untreated *H. pylori*; NC: macrophages (negative control), *: $p < 0.001$ compared to HP. Bars represents the mean ± SD for at least twice independent experiments carried out in triplicate

Proteins expression analysis by two-dimensional electrophoresis

The 2-DE analysis from HP cultured at 0.063 and 0.125 µg/mL of amoxicillin for 24 hours showed 312 spots ranging in molecular mass from 108 to 13 kDa and pIs from 9.08 to 4.14 (Fig. 3A) and 340 spots ranging from 107 to 13.6 kDa and pI from 9.3 to 3.7 (Fig. 3B), respectively. The protein extract from the control experimente obtained after 24 hours of incubation showed 138 spots ranging from 86.5 to 14.5 kDa and pI from 7.2 to 3.9 (Fig. 3C). After analysis by ImageMaster 2D Platinum software (GE Healthcare), we identified 31 proteins whose expression levels changed between the HP and HPAMX conditions (represented by percentage of spot volume).

The spots were excised from the gels and identified by mass spectrometry. Nine spots showed significant identity (score greater than the limit of significance) with HP and homology with other bacteria when tested against a database of eubacteria protein. Proteomics analyses of the proteins selected for HPAMX (Fig. 3, Table 1), revealed that these sub-MICs treatments affected the expression ($p < 0.05$) of heat-shock protein 70 kDa **(**Q9ZMW4/C4LGV8**),** ATP synthase alpha subunit (Q9ZK79/Q8FQ22), heat shock protein 60 kDa (Q9ZN50/ C4LKV2), enolase **(**Q1CV03/C4LHL7**),** argininosuccinate synthase (I0E2L2/C4LIE1), RNA polymerase alpha subunit (Q9ZJT5/Q8NSV2), superoxide dismutase (Q9RM31), neutrophil activating protein (G1UIZ2), and 50S ribosomal protein (C4LL72).



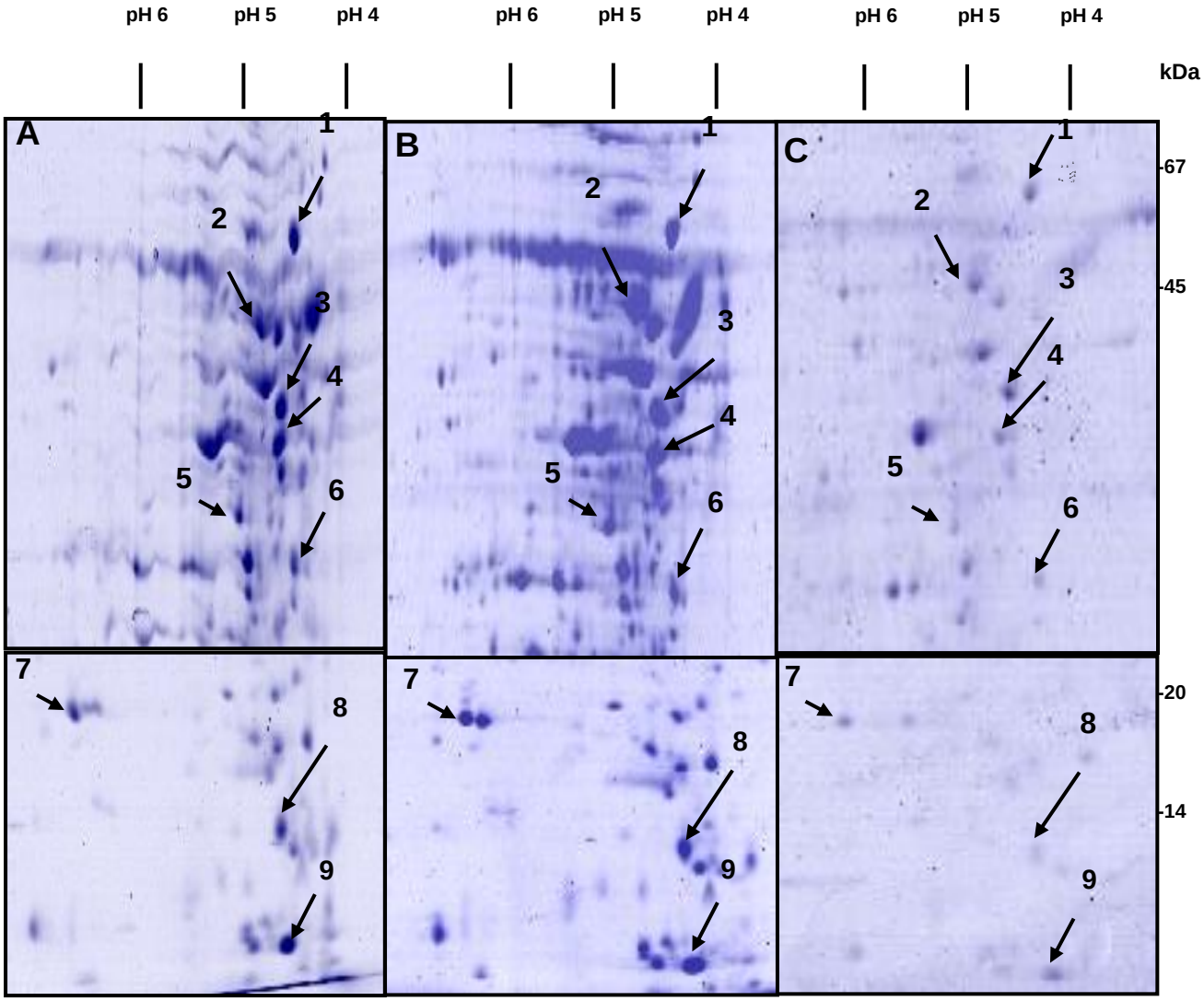


Fig. 3 2-DE maps and screening of protein spots differentially expressed by *Helicobacter pylori* cultured with subinhibitory concentrations of amoxicillin for 24 hours, stained with Coomassie Blue R-250. (A): *H. pylori* cultured with amoxicillin at 0.063 µg/mL (¼ MIC); (B): *H. pylori* cultured with amoxicillin at 0.125 µg/mL (½ MIC); (C): *H. pylori* cultured without amoxicillin; (1) Heat shock protein 70 kDa, (2) ATP synthase alpha subunit, (3) Heat shock protein 60 kDa, (4) Enolase, (5) Argininosuccinate synthase, (6) RNA polymerase alpha subunit, (7) Superoxide dismutase, (8) Neutrophil activating protein, (9) 50S ribosomal protein.

Table.1. Identification and characterization of differentially expressed proteins of *Helicobacter pylori* grown at subinhibitory concentrations of amoxicillin *% volume ratio: percentage volume ratio for each protein obtained from H. pylori cultured with amoxicillin relative from the control; Mw/pI: molecular weight (Mw) and isoelectric point (pI) are presented as experimental values*

SPOT	Protein name	Mw/pI	Differentially expressed protein (% volume, mean±SD)			Protein function[17]
			Amoxicillin concentration (µg/mL)			
			0.063	0.125	Control	
1	Heat shock protein 70 kDa	64.10/4.61	1.75±0.04	1.76±0.05	0.78±0.02	Acts as a chaperone.
2	ATP synthase alpha subunit	54.13/4.92	1.63±0.03	1.58±0.02	1.21±0.02	RNA polymerase alpha subunit.
3	Heat shock protein 60 kDa	49.93/4.73	1.75±0.02	1.45±0.02	0.97±0.04	Prevents misfolding and promotes the refolding and proper assembly of unfolded polypeptides generated under stress conditions.
4	Enolase	44.27/4.76	1.49±0.03	1.34±0.03	0.95±0.04	Catalyzes the reversible conversion of 2-phosphoglycerate into phosphoenolpyruvate. It is essential for the degradation of carbohydrates via glycolysis.
5	Argininosuccinate synthase	41.09/5.11	0.79±0.03	0.77±0.02	0.50±0.02	Transferase activity and tRNA processing and argininosuccinate biosynthesis.
6	RNA polymerase alpha subunit	37.67/4.61	0.84±0.04	0.79±0.03	0.43±0.02	Transcription of DNA into RNA.
7	Superoxide dismutase	21.15/5.80	0.89±0.02	0.83±0.02	0.51±0.01	Destroys radicals which are normally produced within the cells and which are toxic to biological systems
8	Neutrophil activating protein	17.24/4.69	1.10±0.02	0.82±0.01	0.62±0.02	Cellular iron ion homeostasis, response to stress.
9	50S ribosomal protein	13.88/4.59	1.61±0.01	1.70±0.01	0.95±0.02	Connecting to the 23S rRNA and contact with the A and P site tRNA.

DISCUSSION

H. pylori is often neglected for antimicrobial susceptibility testing because of its complex growth requirement and low recovery rate by bacterial culture. [18] Increasing reports of treatment failure have been related to development of antibiotic resistance or poor compliance with the treatment regimen.[3][9] It has been frequently thought that antibiotics, even at subinhibitory concentrations, should necessarily reduce bacterial fitness and, in the case of bacterial pathogens, virulence. [11] [19] Several studies have focused on the interaction between *H. pylori* and the innate immune system showing *H. pylori*-induced cytokine production by antigen-presenting cells. [20] [21] In attempt to elucidate whether amoxicillin affects the immunological properties of *H. pylori*, we assessed cytokine release by RAW 264.7 cells by HPAMX used as stimuli. We showed that HP after being exposed to amoxicillin stimulated IL-1β and IL-12, whereas IL-6 and TNF-α release were not altered. Kranzer *et al*. [21] demonstrated that cytochalasin D reduced *H. pylori*-stimulated IL-12, IL-

1β, and TNF secretion by dendritic cells by blocking phagocytosis, whereas IL-6, IL-8, and IL-10 release remained uninfluenced.

A part from its role in inflammation, IL-1β is a powerful inhibitor of gastric acid secretion. Its effects promote hypochlorhydria, favoring further colonization of *H. pylori* and more severe gastritis.[1] Clinically, it is known that hypochlorhydria predisposes to gastric atrophy. [2] An increased level of IL-12 in gastric mucosa plays a key role in *H. pylori*-associated gastric disease, and is also involved in the induction or amplification of the T-helper type-1 response.[22] Although IL-12 has been shown to be essential for protection against *H. pylori*, overproduction of this cytokine is crucially implicated in the pathogenesis of duodenal ulceration.[23] Our results indicated that amoxicillin at subinhibitory concentration may be involved in amplification of the inflammatory response in *H. pylori* infection.

The stimulatory activity of HPAMX on release of cytokines from RAW 264.7 cells as compared to HP may be related to the bacterial surface changes induced by amoxicillin. Hafsi *et al*. [24] showed that addition of polymyxin B to *H. pylori*-treated dendritic cells did not alter the stimulatory effect in comparison to nontreated *H. pylori*. Polymyxin B disorganizes the cell wall of gram-negative bacteria but leaves most of the bacterial structure untouched. Beta-lactam antibiotics, including amoxicillin, exert their antibacterial effects by inactivating the high-molecular-weight penicillin-binding proteins (PBPs) that are responsible for the final stages of peptidoglycan biosynthesis. [25]

Proteome analysis has been successfully used to identify factors of *H. pylori* that are potentially involved in pathogen-host interactions.[26][27] Amounts of accumulated data support the hypothesis that surface-related proteins are involved in bacterial pathogenesis. Heat-shock proteins (HSPs), also called stress proteins, are a group of proteins present in both prokaryotic and eukaryotic cells.[28] Under normal conditions, HSPs are present at low concentrations in cells, but under stress conditions they accumulate to high levels [29] and therefore enable cells to survive. The protection probably is mediated by the capacity of HSP to function as molecular chaperones to prevent inappropriate protein aggregation and to mediate transport of immature proteins to the target organelles for final packaging, degradation, or repair. [28] Bacterial HSP have been shown to be important immunogens stimulating both T cells and B cells.[30] Two out the nine most over-expressed proteins from HPAMX were the molecular chaperones HSP70 and HSP60, both with adhesion function.[31] It is now clear that HSP60 can activate innate immune receptor resulting in the production of

proinflammatory molecules and factors such as IL-12, IL-15, IL-6, and IL-1β.[32] The increased level of the IL-1β and IL-12 induced by HPAMX can be associated to the increased expression of HSP60.

Proteins that exhibit more than one function is termed moonlighting, and many highly conserved proteins involved in metabolic regulation or the cell stress response have a range of additional biological actions, which are involved in bacterial virulence, such as enolases.[33] Enolase, a glycolytic enzyme, catalyzes the reversible dehydration of 2-phosphoglycerate to phosphoenolpyruvate in one of the steps of the glycolytic pathway.[34] Localized on the cell surface, enolase is one of the best-characterized human plasminogen receptors that promotes the host-pathogen interactions, contributing to the colonization and pathogenesis of this organism.[35] It is possible that the enhancement of HPAMX surface enolase might potentiate *H. pylori* virulence playing an important function in the interaction with the host.

A major proinflammatory factor produced by *H. pylori* is *H. pylori* neutrophil activating protein (HP-NAP). HP-NAP not only recruits leukocytes from the vascular lumen, but also stimulates them to produce messengers that may contribute to the maintenance of the inflammation associated with the *H. pylori*.[36] HP-NAP also promotes Th1 responses by inducing the production of cytokines, such as IL-12 and IL-23, in cells of the innate immune system.[7] Although IL-12 plays a key role in host defense, it has also been implicated in chronic inflammatory disorders characterized by excessive Th1 responses.[7] In this study, we demonstrate that HPAMX is able to up-regulate the HP-NAP expression that contributes to *H. pylori* induced inflammation. The proteomic analysis shows increased expression of argininosuccinate synthase in HPAMX, a typical intermediate of the urea cycle.[37] Although urease is not required for *in vitro* viability of *H. pylori*, it is clear that this enzyme is a critical virulence determinant necessary for colonization of the gastric mucosa.[38] Moreover, the stimulation of production of cytokines by *H. pylori* urease plays a central role in the development of gastroduodenal inflammation.[39]

An important finding from our study is that the superoxide dismutase (SOD), an enzyme that plays a central part in *H. pylori* defense mechanisms against reactive oxygen species [22] was over expressed in the presence of amoxicillin. Mechanisms for detoxification of reactive oxygen species in *H. pylori* are important for *H. pylori* pathogenesis/persistence.[40] The

correlation between *H. pylori* SOD activity and gastric cancer has been recently confirmed.[41] In the clinic, bacteria may be exposed to sub-MICs of antibiotics at the beginning and end of a dosing regimen, between doses, or continuously during low-dose therapy.[42] Our findings may have clinical relevance because amoxicillin is often implemented in *H. pylori* treatment protocols.

CONCLUSION

The results shown in the present work indicate that sub-MICs of amoxicillin can produce specific changes on the cell wall proteins and behavior of susceptible *H. pylori* that can contribute to the pathogenesis of the bacterium.

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