FATE OF *HELICOBACTER PYLORI* ARTIFICIALLY INOCULATED IN LETTUCE AND CARROT SAMPLES

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ABSTRACT

Helicobacter pylori is known, worldwide, as the causative agent of gastric diseases. However, its transmission route has not been completely understood. To evaluate the survival of *H. pylori* (a clinical and a reference strain) artificially inoculated on lettuce and carrot samples, portions of 25 g were inoculated with approximately 10^6 CFU/g of *H. pylori* and packed under normal and/or modified atmosphere (3% oxygen, 10% carbon dioxide, and 87% nitrogen). The inoculated food samples were stored at 8ºC, with daily enumeration of *H. pylori* populations on Columbia blood agar (CBA) and/ or *Helicobacter pylori* Special Peptone Agar (HPSPA). When CBA with antibiotics was used, the clinical isolate *H. pylori* HP1 was detected for up to 72 h in sanitized lettuce and carrot. In sterilized carrot samples, *H. pylori* HP1 remained viable for up to 96 h. The CBA without antibiotics allowed the recovery of *H. pylori* ATCC 43629, from carrot samples stored in both atmospheres tested, for up to 120 hours. Our results reinforce that foodborne transmission of *H. pylori* cannot be disregarded yet.

Key words: *H. pylori*, peptic ulcer, modified atmosphere, vegetable

INTRODUCTION

Helicobacter pylori is a bacterium that is able to colonize the human gastric mucosa and plays an important role in chronic gastritis, gastric/duodenal ulceration and maybe cancer (7). Despite the clinical significance of the diseases caused by *H. pylori*, the transmission routes are not completely understood (8,17,20,21). To date, iatrogenic, oral-oral, and fecal-oral routes are the main postulated modes of transmission. The existence of the fecal-oral route depends partially on the ability of *H. pylori* to survive at least for a limited time in the environment (11,13).

When *H. pylori* is exposed to adverse conditions, its morphology changes from rod shaped viable cells to viable but non-cultivable cocci (VNC). However, the coccoid form is believed to be still infective (4,6).

West *et al.* (23) demonstrated that in the VNC state, *H. pylori* was able to survive in water microcosms for up to one year.

Successful cultivation of *H. pylori* from wastewater stresses the hypothesis that poor quality water could represent an important vehicle for its transmission (1,11,15,16). Epidemiological surveys revealed a positive correlation between infection by *H. pylori* and the consumption of poor hygienic quality water and foods (1,2,9,10). These findings are of special concern for populations with high *H. pylori* prevalence, and are deprived of basic sanitation systems.

Moreover, nowadays it has been observed an increasing demand for minimally processed vegetables packed under modified atmosphere (3). It can be speculated that this kind of packaging will favour the survival of pathogens requiring reduced oxygen concentration for its growth, as is the case of *H. pylori*.

Difficulties in elucidating the transmission routes of *H. pylori* are partially due to unsuitable detection methods available for the recovery of this pathogen from samples with a dense competing microflora (14,17,18). In this work we evaluated

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selective and non-selective media for the recovery of *H. pylori* from vegetable foods.

MATERIALS AND METHODS

Bacterial strains

A clinical isolate of *H. pylori* (designated as *H. pylori* HP1) and *H. pylori* ATCC 43629 were used in this study. The former was isolated from human gastric biopsy at Faculdade de Medicina de Ribeirão Preto - Universidade de São Paulo, Brazil and was kindly donated by Prof. Marcelo Brocchi. The reference strain was obtained directly from American Type Culture Collection (ATCC), USA. The bacterial strains were kept at -70ºC in tubes containing 0.8 ml of Brain Heart Infusion broth (Oxoid, Ltd., Basingstoke, England) with 20% (v/v) glycerol (Merck, KgaA, Darmstadt, Germany). Working cultures were made right before each use.

Inoculum preparation

The content of *H. pylori* stock culture vial was spread on the surface of a Columbia Agar plate supplemented with 5% defibrinated horse blood (CBA) and selective supplement for *H. pylori*, according to Kelly *et al.* (14). The selective supplement contained: 10 mg/l vancomycin, 5 mg/l trimethroprim lactate, 5 mg/l cefsulodin and 5 mg/l amphotericin B (Dent - Oxoid). This medium was named in the present work as modified Columbia Agar. Plates were incubated at 37ºC using an anaerobic jar with a microaerobic atmosphere generator sachet (CampyGen - Oxoid).

A three-day-old culture of *H. pylori* on agar plate was washed with 4 ml of sterile saline (0.85% w/v sodium chloride) to yield a suspension of cells. This suspension was further transferred to a sterile test tube and serial decimal dilutions were prepared. The optical density at 620 nm (spectrophotometer Spectronic 20, Bausch & Lomb, Kyoto, Japan) was obtained for each dilution and the number of CFU/ml was determined by spread plating 0.1 ml of each dilution on CBA and incubating for 24 h at 37ºC, under microaerophilic atmosphere. *H. pylori* used in inoculation experiments was subjected to additional tests such as: urease, catalase, oxidase and Gram staining.

Food samples

Lettuce (*Lactuca sativa*) and carrot (*Daucus carota*) samples were purchased at a retail market in the city of Ribeirão Preto - São Paulo, Brazil, and transported to laboratory under refrigeration.

Lettuce samples

Upon arrival, the lettuce sample was hand washed under tap water, sanitized by dipping in a sodium hypochlorite solution (100 mg/l), rinsed and fractionated into 25 g portions. Subsequently, the fractionated sample was exposed to U.V.

radiation (λ=253.7 nm) for 30 min on each side, in a safety cabinet hood (level II). The residual mesophilic microflora was enumerated by plating 0.1 ml of decimal dilutions of sample prepared in sterile saline (0.85% w/v sodium chloride) on Plate Count Agar (Oxoid). The plates were incubated at 37ºC for 24 h. A control that was only washed (not sanitized) was also analyzed.

Carrot samples

The carrot samples were hand washed under tap water, peeled and fractionated in to 25 g portions. For decontamination, we applied the same treatment used for lettuce samples. Moreover, portions of carrot sample were decontaminated by sterilization in an autoclave at 121ºC for 15 min, to eliminate the interference of autochthonous microflora in selective media.

Artificial inoculation

Portions (25g) of carrot and lettuce samples were individually packed in plastic bags and inoculated with 2.5 ml of *H. pylori* 10^{-1} dilution (prepared as described above) to yield a final population of ca. 10^6-10^7 CFU/g. Uninoculated samples were used as control. The bags containing inoculated and control samples were stored at 8ºC until the enumeration time.

Additionally, we tested the survival time of *H. pylori* ATCC 43629 artificially inoculated in sterilized carrot sample packaged in plastic bags with high barrier to oxygen (Cryovac®, São Paulo, Brazil), filled with a microaerophilic gas mixture comprising 3% O_2, 10% CO_2, and 87% N_2 (Air liquide, São Paulo, Brazil) using Selovac 200B (Selovac, São Paulo, Brazil). Uninoculated samples were used as controls. The inoculated and control samples were stored at 8ºC until the enumeration time.

Evaluation of *H. pylori* survival

Culture media

H. pylori HP1 was recovered in modified CBA, and *H. pylori* ATCC 43629 in CBA and in HPSPA (*Helicobacter pylori* Special Peptone Agar) supplemented with vancomycin (10 mg/l), amphotericin (5 mg/l), cefsulodin (10 mg/l), polymyxin B sulfate (62,000 IU/l), trimethoprim (40 mg/l) and sulfamethoxazole (20 mg/l) (14, 18). HPSPA and Columbia agar without supplements were also tested for the recovery of *H. pylori* ATCC.

Sampling procedure

For the recovery of *H. pylori* HP1 from artificially inoculated lettuce and carrot samples, two bags of each inoculated samples and the control bag were added of 225 ml of saline and homogenized for 1 min, using a Bag Mixer (Interscience, France). Serial decimal dilutions were prepared and each dilution (0.1ml) was spread plated on modified CBA. The plates were incubated at 37ºC in anaerobic jars with microaerophilic atmosphere generator sachet (CampyGen, Oxoid). This procedure was

repeated until the population of *H. pylori* decreased to levels below the detection limit of the method.

The same protocol was followed for the recovery of *H. pylori* ATCC 43629 artificially inoculated in carrot samples. Besides modified CBA, we also used CBA and HPSPA (*Helicobacter pylori* Special Peptone Agar) without antibiotics and HPSPA supplemented with vancomycin (10 mg/l), amphotericin (5 mg/l), cefsulodin (10 mg/l), polymyxin B sulfate (62,000 IU/l), trimethoprim (40 mg/l) and sulfamethoxazole (20 mg/l).

RESULTS

The characterization tests of the *H. pylori* inocula revealed presence of catalase positive, oxidase positive, urease positive and Gram negative rods. The same profile was found for the viable cells of *H. pylori* recovered along all the experiments. *H. pylori* was not detected in any uninoculated samples.

Fig. 1 represents the survival curve of *H. pylori* HP1 in lettuce and carrot samples. At day zero, the population in carrot samples was 1.4×10^7 CFU/g and was reduced to 5.2×10^6 CFU/g after 24h. The number of viable *H. pylori* cells steadily decreased in the following days and at 120 h, it was undetectable (Fig. 1).

When non-sterile carrot was used for inoculation studies, *H. pylori* HP1 remained viable for up to 72 h (Fig. 1). In lettuce

samples, a survival time of 72 h was also observed for *H. pylori* HP1. After this time, the background microflora overgrew *H. pylori*, preventing its detection. It is also possible that *H. pylori* cells converted to VNC state.
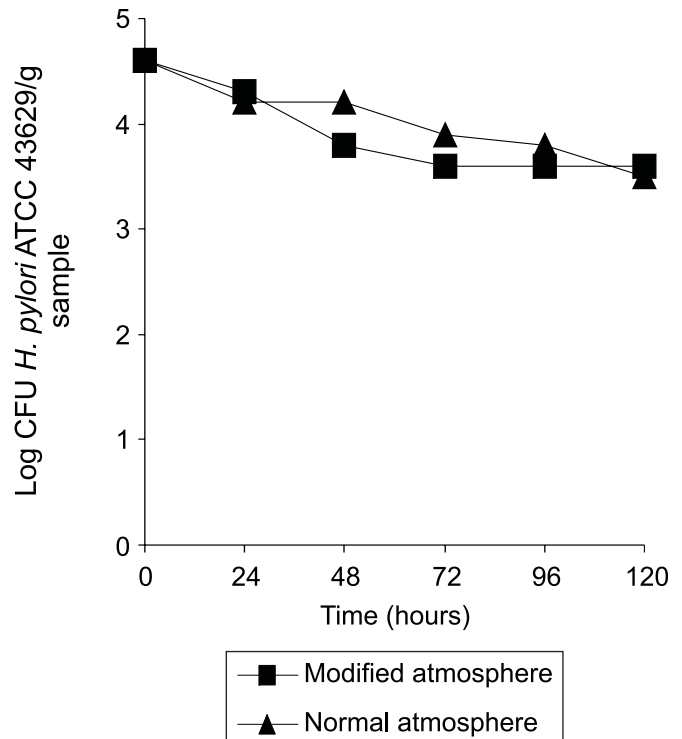
H. pylori ATCC 43629 did not grow on Columbia blood agar or on HPSPA supplemented with antibiotics at any time evaluated. Hence, we carried out further studies with *H. pylori* ATCC 43629 using culture media without selective supplements and sterilized carrot samples.

Fig. 2 shows the survival curve of *H. pylori* ATCC 43629 in sterilized carrot samples packaged under normal and modified atmospheres and stored at 8ºC, using Columbia blood agar supplemented with 5% horse blood. *H. pylori* ATCC 43629 cells were recovered for up to 120 hours from samples stored under both atmospheres. At the end of the experiments *H. pylori* counts decreased 1.0 and 1.1 log in the samples packaged, under normal or modified atmosphere, respectively.

Different results were obtained when the recovery of *H. pylori* ATCC 43629 was done in HPSPA (Fig. 3). We obtained initial counts of 10^3 CFU of *H. pylori* per gram of carrot samples, and within 24 hours the counts dropped bellow the detection limit of the method for samples packaged under normal or modified atmosphere.
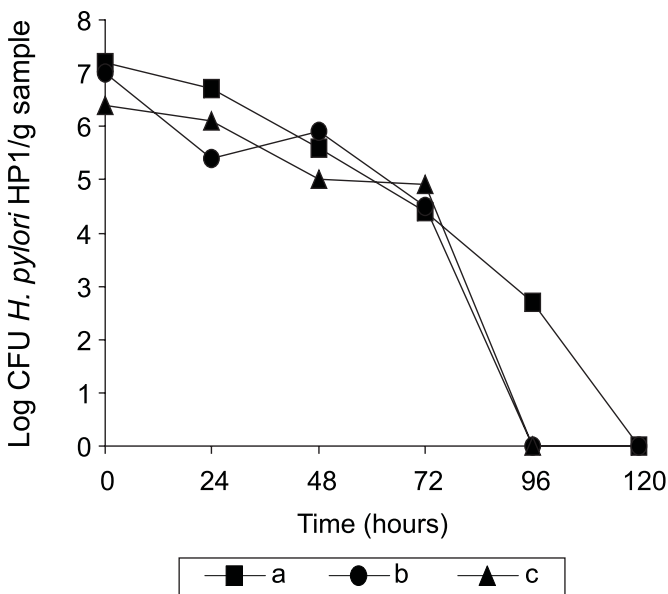
Figure 1. Survival curve of *H. pylori* HP1, artificially inoculated in carrot and lettuce samples stored at 8ºC, enumerated on modified Columbia Agar and incubated at 37ºC. **a:** sterilized carrot sample; **b:** carrot sample sanitized with 100 mg chlorine/l and exposure to UV radiation for 30 min per side; **c:** lettuce sample sanitized with 100 mg chlorine/l and exposure to UV radiation for 30 min per side.

Figure 2. Survival curve of *H. pylori* ATCC 43629 artificially inoculated in sterilized carrot samples stored at 8ºC, enumerated on Columbia blood agar added 5% of horse blood.
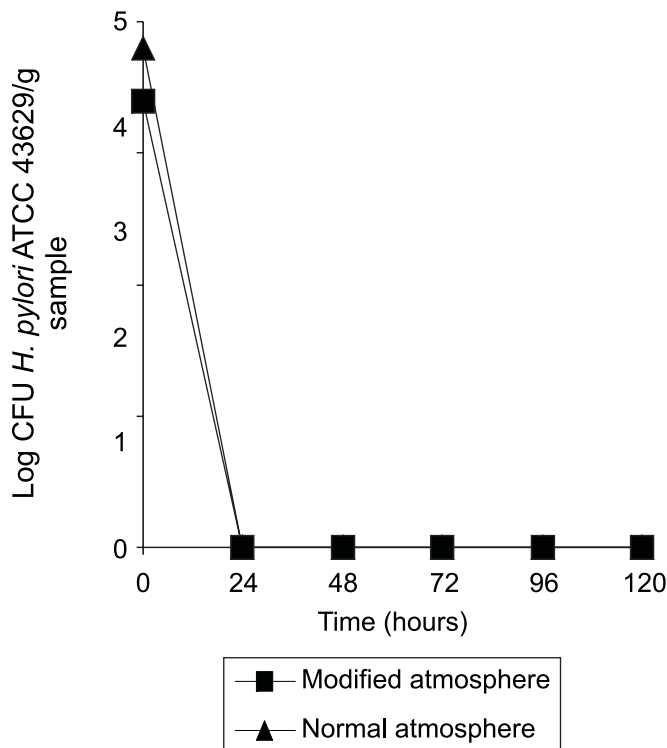
Figure 3. Survival curve of *H. pylori* ATCC 43629 artificially inoculated in sterilized carrot samples stored at 8ºC, enumerated on HPSPA without antibiotics.

DISCUSSION

Several agar media supplemented with horse blood and antibiotics have been employed for the isolation of *H. pylori* from gastric biopsies (22). In this work, modified Columbia agar allowed the detection of *H. pylori* HP1 cells artificially inoculated in selected vegetables. This is in agreement with the findings of Kelly *et al.* (14) who demonstrated the ability of modified Columbia agar to recover *H. pylori* from samples with faster growing autochthonous microorganisms.

H. pylori HP1 lost viability after initial experiments and the results shown are average of duplicates obtained with the same set of experiments. A survival time of 96 h was found for *H. pylori* HP1 in sterile carrot stored at 8ºC. According to Catrenich and Makin (5), the loss in detectability after a initial period of incubation could indicate the conversion of viable *H. pylori* cells to coccoid shape (VNC state).

When non-sterile carrot samples were analyzed, the survival time of *H. pylori* HP1 was shorter (72 h). The colonies observed in plates at 96 h were atypical and were more likely constituents of the psychrotrophic background microflora. This microflora was not detectable in the beginning of the experiment due either to injury induced by the sanitizer or presence in very low numbers.

In agar plates prepared from lettuce samples, besides colonies with morphology typical of *H. pylori,* we also observed atypical ones, but viable *H. pylori* HP1 cells were recovered for up to 72 h. This suggests that the sanitizing treatment was not effective for total inactivation of the background microflora, which impaired *H. pylori* HP1 growth and/or detection after 72 h. Poms and Tatini (17) recovered *H. pylori* NCTC 11638 artificially inoculated in lettuce samples, for up to 48 h, using Wilkins-Chalgren agar as selective plating medium. Those authors hypothesized that the short survival time could be attributed to the faster growth of the background microflora, less susceptible to environmental stresses.

Jiang and Doyle (12) analyzed the survival of *H. pylori* in samples of ground beef. *H. pylori* could be recovered from irradiated ground beef, within 7 days using brain heart infusion broth with 7% horse serum. However, they observed that this pathogen was not recovered from fresh ground beef, because indigenous bacteria outgrew the pathogen since the first 24 hours of experiment.

We observed that modified Columbia agar and HPSPA with antibiotics did not support the growth of *H. pylori* ATCC 43629 and the experiments with this strain had to be carried out with heat sterilized carrot using non-selective media. This could be attributed partially to the high level of genetic diversity presented by *H. pylori* strains, which may cause fail of growth in currently available defined media (19).

Growth of *H. pylori* is better achieved in a microaerophilic atmosphere, since elevated tension of oxygen could be toxic for its growth. So, we analyzed the effect of packaging under a modified atmosphere (very similar to that required for *H. pylori* growth) on the survival time of *H. pylori* ATCC 43629 in carrot samples. Our results (Fig. 2) showed that besides gaseous composition of the environment, other factors should have been decisive for *H. pylori* survival, since similar results were observed for normal and modified atmosphere.

When the recovery of *H. pylori* ATCC 43629 was done in HPSPA without antibiotics, we observed that, since the first 24 hours, this microorganism lost its viability (Fig. 3). Testerman *et al.* (19) reported difficulties when cultivating *H. pylori* in culture media without blood or serum. The authors affirmed that, besides the classical nutritional requirements, blood components would offer other stimulating factors for *H. pylori* growth.

Differences between the survival time of a clinical isolate of *H. pylori* and a reference strain were early reported. West *et al.* (24) obtained diverse results when they compared the effect of physical environmental conditions on the survival of a clinical isolate of *H. pylori* and a reference strain. They affirmed that the difference could be due to repeated subculture, which would lead to a laboratory adaptation and possible *in vitro* chromosomal mutation. Another hypothesis was that *H. pylori* changed to VNC stage.

The results for survival of *H. pylori* ATCC 43629 (Figs. 2 and 3) represent the average of two experiments. A third independent replicate was performed, but the results obtained for each condition in both culture media were unexpectedly different (data not shown).

Similar problems were reported by others researchers. West *et al.* (24) evaluated the effects of physical environment on survival of three strains of *H. pylori* and, reported a variability between repeated experiments in survival times of the same *H. pylori* strain. They affirmed that the variability was either of unknown cause or due to contamination of inocula. Considering that we did not detect contamination of inoculum, we hypothesize the difference we observed could be explained by genetic diversity of *H. pylori* or by its morphological conversion from bacillary to coccoid form.

Jiang and Doyle (12) also performed experiments with *H. pylori* aiming to optimize the detection in foods. The authors reported that, in one of the conditions tested, quite different results were obtained for a repetition of the same experiment.

In conclusion, the role of vegetables eaten raw in the transmission of *H. pylori* cannot be completely discarded, because *H. pylori* is able to survive in foods at least for a limited time. The choice of a culture medium to recover this pathogen from samples with a dense microflora still needs to be carefully studied to prevent underestimation of *H. pylori* populations in food and environmental samples.

ACKNOWLEDGEMENTS

RESUMO

Recuperação de *Helicobacter pylori* artificialmente inoculado em amostras de alface e cenoura

H. pylori é o agente da maioria dos casos de gastrites e úlceras pépticas. Entretanto, sua forma de transmissão não foi completamente elucidada. Para avaliar a sobrevivência de *H. pylori* (uma cepa de referência e uma clínica), artificialmente inoculado em amostras de cenoura e alface, porções de 25g das amostras receberam aproximadamente 10^6 UFC/g de *H. pylori*, e foram embaladas em atmosfera normal e/ou modificada (3% oxigênio, 10% dióxido de carbono, e 87% nitrogênio). Em seguida, foram armazenadas a 8ºC, com enumeração diária da população de *H. pylori* em ágar Columbia sangue (CBA) e/ou *Helicobacter pylori* Special Peptone Agar (HPSPA). Quando usamos CBA com antibióticos, o isolado clínico de *H. pylori* (HP1) foi detectado por até 72 horas nas amostras de alface e cenoura sanitizadas. Em amostras de cenoura esterilizadas, *H. pylori* HP1 permaneceu viável por até 96 horas. CBA sem antibióticos permitiu a recuperação de *H. pylori* ATCC 43629, a partir de amostras de cenoura embaladas em ambas as atmosferas, por até 120 horas. Nossos resultados reforçam que a transmissão de *H. pylori*, através do consumo de água e alimento contaminados, ainda não pode ser descartada.

Palavras-chave: *H. pylori*, úlcera péptica, atmosfera modificada, vegetais

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