EVALUATION OF DIFFERENT CULTURE MEDIA FOR DETECTION AND QUANTIFICATION OF *H. PYLORI* IN ENVIRONMENTAL AND CLINICAL SAMPLES.

Irene Hortelano[a*], Yolanda Moreno[a*], Fidson Juarismi Vesga[c], María Antonia Ferrús[b*].

[a]Research Institute of Water and Environmental Engeneering (IIAMA), Universitat Politècnica de València, 46022 Valencia, Spain

[b]Biotechnology Department, Universitat Politècnica de València, 46022 Valencia, Spain

[c]Microbiology Department, Science Faculty, Pontificia Universidad Javeriana, Bogotá, Colombia.

Corresponding author: Yolanda Moreno. Universitat Politècnica de València, Camino de Vera 14, 46022 Valencia, SPAIN; Tel/fax: +34963877090, Email: ymoren@upvnet.upv.es

ORCID codes:

Irene Hortelano: 0000-0002-8149-3796

Yolanda Moreno: 0000-0003-3688-5157

M. Antonia Ferrús: 0000-0002-7961-9142

Fidson Juarismy Vesga: 0000-0001-9753-3230

Fundings

This work was supported by the Consellería de Educación, Investigación, Cultura y Deporte, of the Community of Valencia, Spain, under project AICO/2018/273, and by the Spanish Ministry of Economy and Competitiveness AGL2014/53875-R Grant.

Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest

Abstract

The objective of the present study was to establish the most suitable culture medium for the isolation of *H. pylori* from environmental and clinical samples.

Ten different culture media were compared and evaluated. Four of them had been previously described and were modified in this study. The rest of the media were designed *de novo*. Three different matrices, tap water, wastewater, and feces, were inoculated with serial dilutions of *H. pylori* NCTC 11637 strain at a final concentration of 10^4 and 10^3 CFU/ml and the recovery rates were calculated.

From inoculated tap water and wastewater samples, *H. pylori* colonies were recovered from four out of the analyzed culture media. When fecal samples were analyzed, the isolation of the pathogen under study was only possible from two culture media.

Different optimal media were observed for each type of sample, even for wastewater and stool samples. Nevertheless, our results indicated that the combination of Dent Agar with polymyxin B sulfate did not inhibit the growth of *H. pylori* and was highly selective for its recovery, regardless of the sample origin. Thus, we propose the use of this medium as a diagnostic tool for the isolation of *H. pylori* from environmental and clinical samples, as well as for epidemiological studies.

Keywords

H. pylori; Isolation; Identification; Culture media; Feces; Environmental samples.

1. Introduction

Helicobacter pylori infection is associated with gastritis, chronic gastritis, peptic and duodenal ulcer, and the organism is also implicated in the development of gastric cancer (Graham et al. 1991), being the only bacteria with a demonstrated carcinogenic action. Bacterial colonization of the gastric mucosa is the main cause of ulcers in the stomach and duodenum (Suzuki et al. 2012).

Although 60% of the world's population is infected with *H. pylori*, its transmission pathway is unclear (Loke et al. 2016). Different routes have been pointed out, such as oral-oral, gastro-oral, fecal-oral, and indirect transmission via water or foods (Sjomina et al. 2018; Atapoor et al. 2014). Some studies have analyzed the potential of water, including drinking water, as an infection source for *H. pylori* (Santiago et al. 2015). The survival of *H. pylori* in biofilms formed in water supply distribution systems has been also proposed as a probable transmission mechanism for these bacteria (Percival et al. 2009).

H. pylori culture is hindered because of its nutritionally fastidious requirements, slow growth, and the presence of viable but non-cultivable (VBNC) forms. The organism requires a complex growth medium and microaerophilic conditions (O_2 between 2% and 5%, and CO_2 between 5% and 10%). In many laboratories, *H. pylori* is usually cultured under high humidity and micro-aerobic gaseous conditions at 37°C and neutral pH (Testerman et al. 2006). Usually, liquid cultures such as Brucella broth (BD), Mueller-Hilton and Brain Heart Infusion (BHI) are supplemented with 2% to 10% calf serum or 0.2% to 1% β-cyclodextrin, antibiotics, amino acids, and vitamins. Fetal bovine serum has been used as culture media supplement in most studies and it remains one of the most influential *in vitro* growth factors (Duque-Jamaica et al. 2010).

Nevertheless, many authors have confirmed that culture methods are not the most appropriate technique to detect this pathogen from biopsies, and affirmed that the low sensitivity of the culture methods can be explained by factors such as small number of microorganisms present in the sample, their death during the manipulation of the samples and the transformation of bacteria to the VBNC form (Adams et al. 2003).

Isolation of *H. pylori* from non-clinical samples (water, wastewater, soils, or vegetables) is even more difficult, mainly due to its nutritional requirements, unavailability of adequate transport media and presence of contaminants (Rizvi et al. 2000). Environmental samples contain a wide variety of microbiota and different interfering compounds; therefore, the successful detection of this pathogen will depend on enrichment culture conditions that stimulate the growth of *H. pylori* and inhibit the growth of other microorganisms (Jiang and Doyle 2002). It has been also suggested that culture media that are too rich in nutrients and could cause a nutritional shock, hampering the growth of *H. pylori* in culture plates (Azevedo et al. 2004).

The difficulty of isolation increases because, after a time subjected to stressful environmental conditions, such as a low nutrient environment (Azevedo et al. 2004), drug supplementation (Bode et al. 1993), pH change (Azevedo et al. 2007), unusual temperature (Nilsson et al. 2002) or extended periods of culture (Sato et al. 2003), *H. pylori* enters the viable but non-culturable state (VBNC), whose changes in cell morphology, cell wall, membrane composition, gene expression and metabolism prevent their growth in culture media (Bai et al. 2016). As these VBNC forms cannot be detected by culture techniques, the potential transmission through environmental reservoirs may be undervalued (Bode et al. 1993; Percival and Suleman 2014).

Thus, isolation of *H. pylori* is very frequently unsuccessful, and there are few reliable studies about the isolation of *H. pylori* from environmental complex samples (Cellini et al. 2014). Moreover, an optimal standard culture media to this aim has not been still developed.

Despite all these disadvantages, culture is the gold-standard method to detect viable *H. pylori* from different environments when comparing with molecular techniques. Furthermore, bacteria isolation is basic for epidemiological and antimicrobial sensitivity studies. Different attempts at culturing the bacteria have been made, using newly designed growth media under different conditions. HP medium, developed by Degnan et al. (2003) to select viable cells from drinking water samples containing mixed microbial populations has been used in seawater samples, but microorganism such as *Vibrio* and *Proteus* were able to grow in it (Fernández et al. 2007).

Azevedo et al. (2004) prepared and compared three different culture media to study a possible nutrient shock effect when recovering *H. pylori* from water samples. The authors concluded that *H. pylori* cells can be stressed in different ways and, for achieving satisfactory recovery rates, it is necessary an adequate nutritional supplementation with amino acids, sodium and potassium chloride, thiamine, iron, zinc, magnesium, hypoxanthine and pyruvate (Testerman et al. 2006) and an incubation atmosphere of 5% CO_2, 5% O_2 and 3% H_2.

Jiang and Doyle (2002) carried out several studies to evaluate the survival and growth of *H. pylori* in enrichment media, and concluded that the addition of supplements such as 0.3% mucin, 0.05% ferrous sulfate, 0.05% sodium pyruvate and 0.008M urea, or adjusting the pH to 5.5 or 4.5 of the enrichment broth, increase the level of the detected microorganisms in enrichment media incubated for 1, 2, 3 and /or 7 days at 37 °C.

Recovery of *H. pylori* from feces is also extremely difficult, as stools are rich in bile salts, hydrolytic enzymes (Van Tongeren et al. 2005) and other commensal biota (Lopes et al. 2014). The addition of polymyxin B sulfate at low concentrations to Columbia Agar supplemented with 10% horse blood and Dent selective supplement (Dent 1988) can reduce the occurrence of mold contaminants (Degnan et al. 2003). Addition of 0.0025% sodium pyruvate also enhances the isolation of *H. pylori* in feces (Moreno et al. 2015).

Another commercial medium, Agar Pylori® (Biomèrieux, France), has been used in some studies, such as the one carried out by Miendje et al. (2010), in which 98 strains were isolated from gastric biopsies. Commercial media plates yielded overgrowth of *Bacillus, Micrococcus luteus* and *Pseudomonas sp.* Moreover, in environmental samples *H. pylori* could not be isolated. Carbone et al. (2005) suggested that this pathogen may require growth factors which are not usually present in commercial culture media.

Thus, the aim of this study was to determine the most suitable culture medium for the isolation of *H. pylori* from both, environmental and clinical samples.

2. Materials and Methods

2.1 Bacteria strain and culture conditions

Reference strain *H. pylori* 11637 (NCTC, UK) was used. The strain was cultured on Blood Agar plates (Blood Agar Base, Pronadisa, Spain) supplemented with 10% (v/v) defibrinated horse blood (Oxoid, UK) and 0.025% (v/v) Sodium Pyruvate (Fisher, USA). The plates were incubated under 90-95% humidity and micro-aerobic conditions (5% oxygen, 10% carbon dioxide, and 85% nitrogen), by using CampyGen™ 3-5L Atmosphere Generation System (Oxoid, UK) in anaerobic jars (Oxoid, UK), at 37ºC for 48 h.

2.2 H. pylori culture media

To determine the optimal medium for *H. pylori* isolation from water and fecal samples, 10 culture media with different nutritional compositions were evaluated. Four of them had been previously described by other authors and were modified in this study. The rest of the media were designed *de novo,* taken into consideration the nutrients that allow the growth of *H. pylori* and inhibit the growth of other microorganisms present in environmental samples. A commercial culture medium was also included:

-Blood Agar Medium (BA): Blood Agar Base (Pronadisa, Spain; composition (g/L): Pancreatic Digestic of Casein (10), Protease peptone N_O (3.5), Yeast Extract (5), Beef heart infusion (3), Corn Starch (1), Sodium Chloride (5) and Agar (15)), 10% (v/v) defibrinated horse blood (Oxoid, ref. SR0050C, UK) and 0.025% (v/v) sodium pyruvate (Fisher, ref. BP356-100, USA).

-Dent Agar (DA): Blood Agar Base, 10% (v/v), defibrinated horse blood, 0.025% (v/v) sodium pyruvate and *H. pylori* Dent Selective Supplement (Oxoid, UK; composition (mg/L): vancomycin (10), trimethoprim (5), cefsulodin (5) and amphotericin B (5)).

-Dent Agar with polymyxin B sulfate (DAPB): Blood Agar Base, 10% (v/v) defibrinated horse blood, 0.025% (v/v) sodium pyruvate, *H. pylori* Dent Selective Supplement (Oxoid, UK) and 2.5 µg/mL of polymyxin B sulfate.

-Culture medium 1 (CM_1), described by Iwamoto et al. (2014), containing BBLTM Brucella Broth (BD, ref.296185, USA; composition (g/L):Pancreatic Digest of Casein(10), Sodium Chloride (5), Peptic Digest of Animal Tissue(10), Sodium Bisulfite (0.1), Yeast Extract (2.0),

Dextrose (1.0)), Agar Bacteriological (Conda, ref 1800; composition (g/L): Agar(15)) and 10% of fetal bovine serum, modified by adding H. pylori Dent Selective Supplement (Oxoid, UK).

-Culture medium 2 (CM_2): CM_1 medium with the addition of 10% (v/v) defibrinated horse blood and 96.7 µg/mL of polymyxin B sulfate.

-Culture medium 3 (CM_3), reported by Ranjbar et al. (2016), containing BBL™ Brucella Broth, 5% (v/v) defibrinated horse, 7% fetal bovine serum, 5mg/L of trimethoprim, 30mg/L of nalidixic acid and 30mg/L of colistin sulfate; modified by adding Dent Selective Supplement (Oxoid, UK) and 250 µg/mL of polymyxin B sulfate.

-BBLS: BBL™ Brucella Agar (BD, ref 211086, USA; composition (g/L): Pancreatic Digestic of Casein (10), Peptic Digest of Animal Tissue (10), Yeast Extract (2), Sodium Chloride (5), Sodium bisulfite (0.1), Dextrose (1) and Agar (15)) supplemented with 5% defibrinated horse blood, Dent Selective Supplement (Oxoid, UK) and 0.4% Isovitalex™ (BD, ref.211875, USA; contains (g/L): Vitamin B_{12} (0.01), L-Glutamine (10), Adenine (1), Guanine Hydrochloride (0.03), *p*-Aminobenzoix Acid (0.013), Nicotinamide Adenine Dinucleotide (0.25), Thiamine Pyrophosphate (0.1), Ferric Nitrate (0.02), Thiamine Hydrochloride (0.003), L-Cysteine Hydrochloride (25.9), L-Cystine (1.1), Dextrose (1000.0)).

-HPA_1: HP Agar designed by Degnan et al. (2003), containing 15 g/L of Agar Bacteriological, 10 g/L of Bacto™ Protease peptone No3, 5 g/L of Bacto™ Yeast Extract, 5 g/L of Beef Extract, 5 g/L of NaCl, 100 mg/L of phenol red, 600 mg/L urea, 0.8 mL/L HCl 1N, 580.03 µg/mL and *H. pylori* Dent Selective Supplemented.

-HPA_2, consisting on HPA_1 supplemented with 25 mg/L of nalidixic acid, streptomycin sulfate and kanamycin, to favor the inhibition of associated microbiota.

-Pylori® Agar (PA) (BioMérieux, France), which is composed (mg/L) by casein Peptone (16), Soy Peptone (7), Meat Extract (0.15), NaCl (6), Agar (15), 100mL of horse plasma and 10 mL of Polyvitalex™ (composition (g/l): Vitamin B_{12} (0.01), L-Glutamine (10), Adenine (1), Guanine Hydrochloride (0.03), *p*-Aminobenzoix Acid (0.013), Nicotinamide Adenine Dinucleotide (0.25), L-Cystine (1.1), Cocarboxylase (0.1), Ferric nitrate (0.02), Thiamine (0.003), Cysteine

Hydrochloride (25.9) and Glucose (0.1)) and a mixture of antibiotics not described by manufacturers.

2.2 Evaluation of culture media

The suitability of the different culture media for the isolation of *H. pylori* was evaluated by recovering serial dilutions of an *H. pylori* NCTC 11637 culture, ranging between 10^5 CFU/mL and 10 CFU/mL. For this purpose, a 48 h *H. pylori* culture on blood agar was used to inoculate 1 mL of PBS (phosphate-buffered saline pH 7.5) to reach a concentration of 10^7 CFU/mL. Counts of total, viable and dead bacteria were carried out by using the Film Tracer™ LIVE/DEAD™ Biofilm Viability Kit (Molecular Probes, USA) according to manufacturer's instructions. Briefly, an aliquot of 125 µL of cell suspension was mixed with 0.4 µL of a mixture of SYTO9 and propidium iodide (1:1), re-suspended and incubated under dark conditions for 5 min at room temperature. A 5 µL aliquot was spotted on a poly-L-lysine (Polysine® slides, Menzel-Glaser, Thermo Scientific) coated slide. The count of total microorganisms was executed under an Olympus epifluorescence microscope (BX50) with U-MWB filter.

Three different matrices, tap water, wastewater, and feces, were inoculated with serial dilutions of the *H. pylori* NCTC 11637 suspension. Previously to the inoculation, samples of tap water and wastewater were sterilized to assure the absence of *H. pylori*. To ensure that feces were negative for this microorganism, qPCR was carried out.

One mL of tap water, wastewater and 1 g of feces were inoculated to obtain a final concentration of 10^4-10^2 CFU/mL. After inoculation, the samples were kept at room temperature for 15 min. Afterwards, aliquots of 100 µL of each inoculated sample were spread onto all the culture media described above and incubated at 37°C under microaerobic conditions for seven days. Plates were examined daily for the presence of colonies. Presumptive *H. pylori* colonies were confirmed by Gram stain, urease catalase and oxidase test. All the tests were performed by triplicate and the results were expressed as the arithmetical median of counts.

3. Results

When dilutions of the pure culture of the reference strain *H. pylori* NCTC 11637 was directly spread onto the plates, bacterial growth and visible and identifiable colonies were observed in seven of the ten media tested. No growth was observed in CM_1, CM_2 and HPA_2 plates at any concentration. Thus, these media were discarded for the rest of the assays.

H. pylori colonies from the dilutions containing 10^5 CFU/mL and 10^4 CFU/mL were recovered from the rest of culture media at the same concentration levels, except for HPA_1, in which counts resulted hundred-fold lower than the original inoculation.

No colonies were recovered from the dilutions containing 10^3 and 10^2 CFU/mL on both, BA and HPA_1 media. From the *H. pylori* inoculum of 10^3 CFU/mL, ten-times less cells grew on DA with 360 CFU/mL, 470 CFU/mL on DAPB, 177 CFU/mL on CM_3, 2750 CFU/mL on BBLS and 3080 CFU/mL on PA medium.

From the lower *H. pylori* dilution (10^2 CFU/mL), 180 CFU/mL were recovered on DA; 107 CFU/mL on DAPB; 50 CFU/mL on CM_3; 590 CFU/mL on BBLS and 510 CFU/mL on PA media.

Tap water samples were inoculated at a final concentration of 10^4 and 10^3 CFU/mL. Growth of *Helicobacter pylori* colonies was observed after 3 days of incubation period in most of the media: DA yielded the best results, recovery 10^4 and 10^3 CFU/mL, respectively. The counts from the rest of the media were lower. From the tap water inoculated with 10^4 cells/mL, PA yielded 10^3 CFU/mL; DAPB, 1770 CFU/mL and a medium of 1150 CFU/mL was obtained on BBLS medium. From the 10^3 CFU/mL dilution, CFU were recovered on DA; 1300 CFU/mL on DAPB; 90 CFU/mL on BBLS and 2420 CFU/mL on PA media. No growth was observed in CM_3 and HPA_1 plates at any concentration.

Final concentrations of *H. pylori* in inoculated wastewater samples reached 10^3 and 10^2 CFU/mL. In all the plates *Helicobacter* cells grew slowly and an incubation period of 15 days was necessary to observe colonies on the media. On CM_3 and DA media, no *H. pylori* colony grew from any dilution. From the sample inoculated with 10^3 CFU/mL, a total of 570 CFU/mL was recovered on DAPB plates; 980 CFU/mL on BBLS; 230 CFU/mL on HPA_1 and 2100 CFU/mL on PA. When plates were inoculated with the 10^2 CFU/mL dilution, only positive results were

obtained from BBLS culture media, with a total of 40 CFU/mL; the rest of culture media were negative.

Fecal samples were inoculated at a final concentration of 10^4 and 10^3 CFU/mL. *Helicobacter pylori* colonies were not observed until 7 days of incubation. From the sample inoculated with 10^4 CFU/mL, the same concentration was recovered from DA and DAPB plates. On DA medium, 1750 CFU/mL and on DAPB medium, 2380 CFU/mL were counted from the samples inoculated with 10^3 CFU/mL of *H. pylori*. For both concentrations, CM_3 and HPA_1 showed unspecific bacterial growth, and therefore were considered non-specific for the isolations of *Helicobacter pylori.* For PA media, in both concentrations under study the growth of *H. pylori* was inhibited.

4. Discussion

Isolation of *H. pylori* from environmental samples is difficult, due to its demanding nutritional requirements, slow growth and sensitivity to toxic forms of oxygen (Adams et al. 2003; Cellini et al. 2004; Azevedo et al. 2004; Santiago et al. 2015). Many authors have reported that cultural methods are extremely limited for *H. pylori* detection, mainly due to the absence of an optimal selective culture medium. Even in biopsy samples, where it is in high concentration and there is no competitive biota, the success rate is highly variable (Al-Sulami et al. 2012; Wang et al. 2015).

H. pylori is a bacterium that grows relatively slowly, and usually requires about 4 days to develop distinguishable colonies. In the present study, we have observed that the incubation period may be longer, depending on the culture medium used and the origin of the sample. In wastewater and inoculated fecal samples, bacterial growth only occurred after 15 and 7 days of incubation, respectively.

In this study, 10 culture media were used to verify their suitability for the isolation of *H. pylori* from environmental samples. Different nutritional supplements and antibiotics were used to prevent the classic microbiological problem of finding a nutritionally rich culture medium that allows *H. pylori* resuscitation and growth, and at the same time can inhibit the

growth of other competitive microorganisms. The media were tested for recovery of *H. pylori* from in tap water, wastewater, and feces.

No correlation between successful cultivation and main nutritional bases, such as peptone or protein extracts was found. CM_1 and CM_2, modified from the liquid culture used by Iwamoto et al. (2014) for the conservation of *H. pylori* colonies isolated from gastric biopsy, as well as HPA_2, modified from the culture medium used by Degnan et al. (2003) for the isolation of *H pylori* in water samples, were not adequate for the culture of *H. pylori*, as no colony was recovered when dilutions up to 10^4 CFU/ml were spread onto the plates. Results were negative, maybe due to the amount of fetal bovine serum used. Studies carried out by Dent et al. (1988), Ansorg et al. (1991) and Testerman et al. (2006) suggested that fetal bovine serum (FBS) can increment the permeability of the outer membrane, increasing the sensitivity to certain antibiotics such a cefsulodin, polymyxin B and thus inhibiting the growth of *H. pylori*. Shibayama et al. (2006) observed a significant inhibitory effect of FBS in liquid media, which they related to the bactericidal effect of complement in serum. However, there are not conclusive evidences that 10% fetal calf serum may be inhibitory in solid media and serum is a usual component of high nutritional media for fastidious microorganisms (Lagier et al. 2015). Therefore, some other aspect of the media composition could be responsible for the observed growth failure.

HPA_2 is probably too nutritious for a microorganism that under natural conditions must adapt physiologically to low nutrient conditions. Consequently, a condition described by Reasone et al. (1985) as a nutrient shock may occur. The conjunction of this shock, with the presence in the media of antibiotics such as nalidixic acid, streptomycin sulfate and kanamycin probably caused an inhibitory effect on *H. pylori*, delaying or suppressing the growth.

A limitation of this study is that CampyGen™ 3-5L Atmosphere Generation System was used for the growth of *H. pylori*, instead off a tri-gas incubator. A damage factor as oxygen toxicity should be considered when CampyGen gas system is used, because the generation of a proper atmosphere takes about half an hour to be achieved (Azevedo et al. 2004). During this period time *H. pylori* cells can be exposed to oxidative stress, which is known to be one of the

298 factors that induce its transformation into the viable but not cultivable state (VBNC) (Nilsson
299 et al. 2002), thus hindering the isolation of the pathogen onto culture media. Although the
300 use of microareophilic generation systems is the only option in many laboratories, this point
301 should be carefully considered when trying to isolate the microorganism from environmental
302 samples.

303 Regarding CM_3, negative results in environmental samples may be due to the presence of
304 colistin. It has reported that, at high concentrations, this antibiotic can inhibit the growth of
305 *H. pylori* (McGee et al. 2011).

306 Several studies have shown that the recovery percentage of *H. pylori* from tap water by
307 culture is very low, thus confirming that culture is not the most appropriate technique to
308 assess the safety of drinking water with respect to this pathogen (Giao et al. 2008; Al-Sulami
309 et al. 2012). Bahrami et al. (2013) investigated 50 samples of tap water from Iran and only
310 two of them (4%) yielded positive culture results by using Brucella Agar medium, while PCR
311 detection of the ureC gen showed that 14 (7%) of water samples were contaminated with the
312 pathogen.

313 In our study, when tap water samples were inoculated, the culture media that allowed the
314 isolation and identification of *H. pylori* were DA, DAPB, BBLS and PA. All of them contain Dent
315 supplement, composed of four antibiotics (Vancomycin, Trimethoprim, Cefsulodin and
316 Amphotericin B), recommended for clinical samples. DAPB included also Polymyxin B,
317 because of its ability to suppress large number of *Pseudomonas spp*, and sodium pyruvate,
318 which improves the growth and aerotolerance of *H. pylori* to neutralize the toxic effects of
319 oxygen, as well as neutralizes the inhibitory effects of antibiotics (Jiang et al. 2002). BBLS was
320 composed by the addition of Isovitalex^{TM} supplement, which provides vitamins, amino acids,
321 coenzymes, glucose, ferric ions, and other factors that allow for the growth of *H. pylori*
322 (Hultén et al. 1998). Finally, PA is a commercial culture media, whose efficacy for primary
323 isolation of *H. pylori* from gastric biopsies and environmental samples has been previously
324 reported (Miendje et al. 2010 and Carbone et al. 2005).

There are few studies about the isolation of *H. pylori* in wastewater. Only the investigation carried out by Lu et al. (2002) demonstrated the cultivability of *H. pylori* in wastewater after immunomagnetic capture. Another studied performed by Moreno and Ferrús (2012) demonstrated the presence of cultivable *H. pylori* in wastewater treatment plants, although *H. pylori* cells could not be completely isolated, due to the growth of competitive biota on the selective agar.

For these samples, our results showed that the most suitable media for the isolation and identification of *H. pylori* were DAPB, BBLS, HPA_1 and PA. HPA_1 (Degnan et al. 2003) contains hydrochloric acid and urea. These results agree with previous studies which suggested that survival of *H. pylori* improves at low pH values with the presence of urea in amounts less than 10mM and that the use of this chemical compounds facilitates the growth and isolation of the microorganism (Stevenson et al. 2000; Jiang et al. 2002; Boro et al. 2016).

The isolation of *H. pylori* from stools is very difficult, due to the complex nature of the sample, rich in bile salts and hydrolytic enzymes (van Tongeren et al. 2005), the presence of competitive biota (Moreno and Ferrús 2012; Lopes et al. 2014; Moreno et al. 2015) and the probable transformation of the microorganism into the VBNC state, due to the environmental stress through the gastro intestinal tract.

When inoculated fecal samples were analyzed, our results showed that the culture media that best allowed for the isolation of *H. pylori* were DA and DAPB. This is an important result since diagnostic of *H. pylori* infection from fecal samples consists only in the detection of *H. pylori* antigens. However, culturing the organism gives much more information, as it allows for the identification of viable, and thus potentially infective bacteria. Moreover, antibiograms can be performed and resistance to antibiotics detected, thus avoiding the use of invasive diagnostic methods or failures in treatment (Moreno et al. 2015).

In our study, different optimal media were observed for each type of sample, even for wastewater and stool samples (PA and DAPB, respectively). This unexpected result may be due to their different composition. Wastewater contains a great variety of toxic chemicals, including antibiotics, detergents, or organic compounds. In our geographical area, emergent pollutants of different chemical groups, such as pharmaceuticals, antibiotics, pesticides, drugs of abuse or perfluorinated compounds, among

others, are usually present (Álvarez-Ruiz et al. 2018). There is no evidence that any of the the ingredients in the successful media might be better at absorbing or detoxifying contaminants than those in the unsuccessful media.

On the other hand, during wastewater treatment a disinfection step with chlorine and/or UV is performed, what can greatly stress *H. pylori* cells. The presence of chemical toxic contamination and the disinfection treatments may explain the prolonged growth period required for *H. pylori* recovery from this type of samples.

The culture media described in this paper were designed and tested for the isolation of *H. pylori* from environmental samples. Due to the nature of this microorganism, the determination of selective agents that inhibit large number of contaminant microbiota, while allowing *H. pylori* successful growth is extremely difficult. Nonetheless, positive results have been obtained regardless of the origin of the sample, by using DAPB, which includes *H. pylori* Dent Selective Supplement, 2.5µg/L of polymyxin B and sodium pyruvate. This combination does not inhibit the growth of *H. pylori* and is highly selective for its recovery. The culture of samples in this medium could be used as a diagnostic tool as well as for epidemiological studies.

References

Adams BL, Bates TC, Oliver JD (2003) Survival of *Helicobacter pylori* in a Natural Freshwater Environment. Appl Environ Microbiol 69: 7461-7466. https://doi.org/10.1128/AEM.69.12.7462–7466.2003

Al-Sulami AA, Al-Edani TA, Al-Abdulaz AA (2012) Culture Method and PCR for the Detection of *Helicobacter pylori* in Drinking Water in Basrah Governorate Iraq. Gastroenterol Res Pract 12: 5. https://doi.org/10.1155/2012/245167.

Álvarez-Ruiz R, Cuñat A, Picó Y (2018) Pollution fingerprinting of sewage sludge and their related risk in Albufera's wetland, Valencia. 16th IWA International Conference on Wetland Systems for Water Pollution Control. http://hdl.handle.net/10261/184328

Ansorg R, Von Recklinghausen G, Pomarius R, Schmid EN (1991). Evaluation of techniques for isolation, subcultivation and preservation of *Helicobacter pylori*. J Clin Microbiol 29: 51-53.

Atapoor S, Safarpoor Dehkordi F, Rahini E (2014) Detection of *Helicobacter pylori* in various types of vegetables and salads. Jundishapur J Microbiol 7(5). e10013.

https://doi.org/10.5812/jjm.10013.

Azevedo NF, Almedia L, Cerquerias S, Dias C, Keevil W, Vieira MJ (2007) Coccoid form of *Helicobacter pylori* as a morphological manifestation of cell adaptation to the environment. Appl Environ Microbiol 73(10): 3423-7. https://doi.org/10.1128/AEM.00047-07

Azevedo NF, Pacheco AP, Keevil CW, Vieira MJ (2004) Nutrient shock and incubation atmosphere influence recovery of culturable *Helicobacter pylori* from water. Appl Environ Microbiol 40: 490-493. https://doi.org/10.1128/AEM.70.1.490-493.2004.

Bahrami AR, Rahimi E, Ghasemian Safaei H (2013) Detection of *Helicobacter pylori* in City Water, Dental Units' Water, and Bottled Mineral Water in Isfahan, Iran. Sci World J 5. https://doi.org/10.1128/AEM.00827-0810.1155/2013/280510.

Bai X, Xi C, Wu J (2016) Survival of *Helicobacter pylori* in the wastewater treatment process and the receiving river in Michigan, USA. J Water Health 14: 692-698. https://doi.org/10.2166/wh.2016.259.

Bode G, Mauch F, Malfertheiner P (1993) The coccoid forms of *Helicobacter pylori*. Criteria for their viability. Epidemiol Infect 111: 483-490. https://doi.org/10.1017/S0950268800057216.

Boro S, Sarma M, Sarma P (2016). *Helicobacter pylori* and Steps for its Elimination: A Review Global J Med Res (F) 16: 31-36.

Carbone M, Maugeri TL, Gugliandolo C, La Camera E, Biondo C, Fera MT (2005) Ocurrence of *Helicobacter pylori* DNA in coastal environment of southern Italy (Straits of Messina). J Appl Microbiol 98: 768-774. https://doi.org/10.1111/j.1365-2672.2004.02517.x.

Cellini L (2014) *Helicobacter pylori*: a chameleon-like approach to life. World J Gastroenterol 20: 5575-5582. https://doi.org/10.3748/wjg.v20.i19.5575.

Degnan AJ, Sonzogni WC, Standridge JH (2003) Development of a plating medium for selection of *Helicobacter pylori* from water samples. Appl Environ Microbiol 69: 2914-2918. https://doi.org/10.1128/AEM.69.5.2914-2918.2003.

Dent JC, McNulty CA (1988). Evaluation of a new selective medium for *Campylobacter pylori*. Eur J Clin Microbiol Infect Dis 7: 555-558. https://doi.org/10.1007/bf01962615

Duque-Jamaica R, Arévalo-Galvis A, Poutou-Piñales RA, Trespalacios AA (2010) Sequential Statistical Improvement of the Liquid Cultivation of *Helicobacter pylori*. Helicobacter 15: 303-312. https://doi.org/10.1111/j.1523-5378.2010.00763.x

Farhat Rizvi, Abdul Hanna (2000) Evaluation of Different Transport and Enrichment media for the Isolation of *Helicobacter pylori.* JAMC 12: 31-33.

Fernández M, Contreras M, Suárez P, García-Amado MA (2007) Use of HP selective medium to detect *Helicobacter pylori* associated with other enteric bacteria in seawater and marine molluscs. Let Appl Microbiol 45: 213-218. https://doi.org/10.1111/j.1472-765X.2007.02174.x

Fujio Sato F, Saito N, Konishi K, Shoji E, Kato M, Takeda H, Sugiyama T, Asaka M (2003) Ultrastructural observation of *Helicobacter pylori* in glucose-supplemented culture media. J Med Microbiol 52: 675-679. https://doi.org/10.1099/jmm.0.05146-0

Giao MS, Azevedo NF, Wilks SA, Vieira MJ, Keevil CW (2008) Persistence of *Helicobacter pylori* in Heterotroplhic Drinking Water Biofilms. Appl Environ Microbiol 5898-5904. https://doi.org/10.1128/AEM.00827-08.

Graham DY (1991) *Helicobacter pylori*: Its epidemiology and its role in duodenal ulcer disease. J Gastroenterol Hepatol 6:105-113. https://doi.org/10.1111/j.1440-1746.1991.tb01448.x

Hultén K, Enroth H, Nyström T, Engstrand L (1998) Presence of *Helicobacter* species DNA in Swedish water. J Appli Microbiol 85: 282-286. https://doi.org/10.1046/j.1365-2672.1998.00500.x

Iwamoto A, Tanahashi T, Okada R, Yoshida Y, Kikuchi K, Keida Y, Yoshida M (2014) Whole-genome sequencing of clarithromycin resistant *Helicobacter pylori* characterizes unidentified variants of multidrug resistant efflux pump genes. Gut Pathog 6: 27. https://doi.org/10.1186/1757-47496-27

Jiang X, Doyle MP (2008) Growth Supplement for *Helicobacter pylori*. J Clin Microbiol 38: 1984-1987.

Jiang X, Doyle MP (2002) Optimizing enrichment culture conditions fordetecting *Helicobacter pylori* in foods. J Food Protect 65: 1949-1954. https://doi.org/10.4315/0362-028x-65.12.1949

Lagier JC, Edouard S, Pagnier I, Mediannikov O, Drancourt M, Raoult D (2015) Current and Past Strategies for Bacterial Culture in Clinical Microbiology. Clin Microbiol Rev. 28(1): 208–236.

Loke MF, Ng CG, Vilashni Y, Lim J, Ho B (2013) Understanding the dimorphic lifestyles of human gastric pathogen *Helicobacter pylori* using the SWATH-based proteomics approach. Sci Rep 6(26784). https://doi.org/10.1038/srep26784

Lopes AI, Vale FF, Oleastro M (2014) *Helicobacter pylori* infection-recent developments in diagnosis. World J Gastroenterol 20: 9299-9313. https://doi.org/10.3748/wjg.v20.i28.9299.

Lu Y, Redlonger TE, Avitia R, Galindo A, Goodman G (2002) Isolation and genotyping of *Helicobacter pylori* from untreated Municipal Wastewater. Appl Environ Microbiol 68 (3): 1436-1439.

McGee D, Geore S, Trainor E, Hortonm K, Hildebrandt E, Testerman L (2011) Cholesterol Enhances *Helicobacter pylori* Resistance to Antibiotics and LL-37. Antimicrob Agents Ch. https://doi.org/10.1128/AAC.00016-11.

Miendje Deyi VY, Van den Borre C, Fontaine V (2010) Comparative evaluation of 3 selective media for primary isolation of *Helicobacter pylori* from gastric biopsies under routine conditions. Diagn Micr Infec Dis 68: 474-476. https://doi.org/10.1016/j.diagmicrobio.2010.08.009.

Moreno Y, Ferrús MA (2012) Specific detection of cultivable *Helicobacter pylori* cells from wastewater treatment plant. Helicobacter 17:327-32. https://doi.org/10.1128/AEM.00827-0810.1111/j.1523-5378.2012.00961. x.

Moreno Y, Pérez R, Ramirez MJ, Calvet X, Santiago P, Ferrús MA (2015) Rapid identification of Viable *H.pylori* Cells in Feces by DVC-FISH. JSM Gastroenterol Hepatol 3: 1049.

Nilsson HO, Blom J, Abu-Al-Soud W, Ljung AA, Andersen LP, Wadstrom T (2002) Effect of Cold Starvation, Acid Stress, and Nutrients on Metabolic Activity of *Helicobacter pylori*. Appl Environ Microbiol 68: 11-19. https://doi.org/10.1128/AEM.68.1.11-19.2002

Percival S, Suleman L (2014) Biofilms and *Helicobacter pylori*: Dissemination and persistence within the environment and host. World J Gastrointest Pathophysiol 5: 122-132. https://doi.org/10.4291/wjgp.v5.i3.122.

Percival S, Thomas JG (2009) Transmission of *Helicobacter pylori* and the role of water and biofilms. J Water Health 7: 469-477. https://doi.org/10.2166/wh.2009.070.

Ranjbar R, Khamesipour F, Jonaidi-Jafari N, Rahimi E (2016) *Helicobacter pylori* isolated from Iranian drinking water: vacA, cagA, iceA, oipA and babA2 genotype status and antimicrobial resistance properties. FEBS Open Bio 6: 433-441. https://doi.org/10.1002/2211-5463.12054.

Reasoner DJ, Geldreich EE (1985) A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol 49: 1-7.

476 Santiago P, Moreno Y, Ferrús MA (2015) Identification of viable *Helicobacter pylori* in drinking
477 water supplies by cultural and molecular techniques. Helicobacter 20: 252-259.
478 https://doi.org/10.1111/hel.12205

479 Shibayama K, Nagasawa M, Ando T, Minami M, Wachino J, Suzuki S, Arakawa Y (2006)
480 Usefulness of Adult Bovine Serum for *Helicobacter pylori* Culture Media. J Clin Microbiol
481 44(11): 4255–4257

482 Sjomina O, Pavlova J, Niv Y, Leja M (2018) Epidemiology of *Helicobacter pylori* infection.
483 Helicobacter e12514. https://doi.org/10.1111/hel.12514

484 Stevenson TH, Lucia LM, Acuff GR (2000). Development of a selective medium for isolation of
485 *Helicobacter pylori* from Cattle and Beef samples. App Environ Microbiol 66: 723-727.

486 Suzuki R, Shiota S, Yamoka Y (2012) Molecular epidemiology, population genetics, and
487 pathogenic role of *Helicobacter pylori*. Infect Genet Evol 12: 203-2013.
488 https://doi.org/10.1016/j.meegid.2011.12.002

489 Testerman TL, Conn PB, Mobley HLT, McGee DJ (2006) Nutritional requirements and
490 antibiotic resistance patterns of *Helicobacter* species in chemically defined media. J Clin
491 Microbiol 44: 1650-1658. https://doi.org/10.1128/JCM.44.5.1650-1658.2006.

492 Van Tongeren SP, Slaets JP, Harmsen HJM, Welling GW (2005) Fecal microbiota composition
493 and frailty. Appl Environ Microbiol 71: 6438-6442. https://doi.org/10.1128/AEM.71.10.6438-
494 6442.2005.

495 Wang YK, Kuo FC, Liu CJ, Wu MC, Shih HY, Wang SS, Wu JY, Kuo CH, Huang YK, Wu DC (2015)
496 Diagnosis of *Helicobacter pylori* infection: Current options and developments. World J
497 Gastroenterol 21: 11221-1235. https://doi.org/10.3748/wjg.v21.i40.11221.

498

499

Table 1. Summary of *Helicobacter pylori* recovery rates from all tested culture media for each of the matrices and dilutions

Culture media	Pure culture				Tap water		Wastewater		Feces	
	10^5 CFU/mL	10^4 CFU/mL	10^3 CFU/mL	10^2 CFU/mL	10^4 CFU/mL	10^3 CFU/mL	10^3 CFU/mL	10^2 CFU/mL	10^4 CFU/mL	10^3 CFU/mL
BA	10^5	10^4	NG	NG	NG	NG	NG	NG	NG	NG
DA	10^5	10^4	360	180	10^4	1490	NG	NG	10^4	1750
DAPB	10^5	10^4	470	107	1770	1300	570	NG	10^4	2380
CM_3	10^5	10^4	177	50	NG	NG	NG	NG	NG	NG
BAS	10^5	10^4	2750	590	1150	90	980	40	NG	NG
HPA_1	1180	160	NG	NG	NG	NG	230	NG	NG	NG
PA	10^5	10^4	3080	510	10^3	2420	2100	NG	NG	NG

*NG: No Growth