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# Laboratory Maintenance of *Helicobacter* Species

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## Abstract

*Helicobacter* species are Gram-negative bacteria that colonize the gastric or intestinal mucosa of many mammalian and avian hosts and induce histologic inflammation. The association of *H. pylori* with gastritis, peptic ulcer disease, and gastric cancers makes it a significant human pathogen. Animal models for these diseases are being used to explore the pathogenesis of *H. pylori* infection and in vaccine development (*UNIT 8B.1*). Both bacterial and host factors contribute to *Helicobacter* pathogenesis, and therefore the microbiology is very important. This unit describes how to culture the most commonly used gastric *Helicobacter* species, *H. pylori, H. mustelae*, and *H. felis*.

## Keywords

*Helicobacter*; growth curve; morphological identification; blood agar preparation; pinch biopsy culture methods

## Subject Group

Microbiology; Gram Negatives; Cell Culture; Bacteria

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In vitro, *Helicobacter* species grow quite slowly and only under conditions of reduced (but not absent) oxygen (i.e., microaerophilic conditions) and rich media. Primary cultures may take up to 5 to 7 days to achieve optimal growth, although subsequent passages take only a few days. Identification of *H. pylori* can be made by confirming the presence of urease, catalase, and oxidase enzymes, and by observing microscopic evidence of high motility and a spiral or curved morphology (Windsor and O'Rourke, 2000). Colonies are small and round, and appear translucent against the blood agar background. *H. pylori* is resistant to sulfonamides, vancomycin, trimethoprim, and cefsulodin, which allows for the creation of a very effective selective media when isolating bacteria from clinical biopsies. It is sensitive, however, to many common antibiotics including tetracycline, gentamicin, kanamycin, and

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**Internet Resources**

Web sites for detailed information about H. pylori history, epidemiology, and pathogenesis.

http://www.helico.com

http://digestive.niddk.nih.gov/ddiseases/pubs/hpylori/index.aspx

Biosafety in Microbiology and Biomedical Laboratories 5$^{th}$ Edition, December 2009. Contains details for handling bacteria of differing safety levels and separate sections for individual pathogens such as H. pylori. PDF versions of the entire monograph, as well as individual sections are available from the U.S. Centers for Disease Control at the following link:

http://www.cdc.gov/biosafety/publications/bmbl5/

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penicillin. These antibiotics may therefore be used to kill extracellular *H. pylori* when performing co-culture experiments with phagocytes, or as selection markers when introducing gene cassettes into the *H. pylori* genome.

In the present unit, protocols for growing *Helicobacter* organisms on plates (Basic Protocol 1) or in liquid cultures (Basic Protocol 2) are presented first, followed by Basic Protocol 3, which describes the use of agar stabs to culture *Helicobacter*. The culturing of *H. pylori* from clinical biopsy specimens is covered in Basic Protocol 4.. Support protocols are presented that describe the preparation of solid media for growth on plates (Support Protocol 1), a method for quantifying numbers of *Helicobacter* organisms in an actively growing culture (Support Protocol 2), and techniques for quantifying *H. felis* in vitro (Support Protocol 3). Support Protocol 4 describes the quantification of Helicobacter using quantitative PCR. Additionally, a method for liquid culture of *Helicobacter* has been provided that does not require the use of a tissue culture incubator (see Alternate Protocol). Finally, methods are provided for confirming growth of *H. pylori* (see Support Protocol 5).

*CAUTION: H. pylori* and *H. mustelae* are Biosafety Level 2 (BSL-2) pathogens; *H. felis* is a BSL-1 pathogen. Adult acquisition of *H. pylori* is thought to be relatively rare, and most infected individuals remain asymptomatic and healthy for life. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See *UNIT 1A.1* and other pertinent resources (*APPENDIX 1B*) for more information.

## STRATEGIC PLANNING

Several different *Helicobacter* selective media supplements have been described (see Table 8B.1.1). Skirrow's recipe, available commercially from Difco, BBL, and Oxoid, was originally developed for isolation of *Campylobacter* species. Although Skirrow's supplement (10 μg/ml vancomycin, 5 μg/ml trimethoprim, and 2.5 IU/ml polymyxin B) plus amphotericin B has been used for isolation and culture of *Helicobacter* species, the authors have found the recipes in Table 8B.1.1 to be more effective. In particular, the so-called Glaxo selective supplement A (GSSA) is useful for the isolation of *H. pylori* from mice when attempting to adapt a new *H. pylori* strain to mice (Lee et al., 1997; McColm, 1997; McColm et al., 1995). Note, however, that use of GSSA could induce a bias into the selection/adaptation process, as some *H. pylori* isolates have been shown to be sensitive to nalidixic acid. One or the other of the supplements must be used when attempting to isolate *H. pylori* or one of the other *Helicobacter* species from a human or animal biopsy. This is particularly important for isolating bacteria from mice since they are coprophagic (they consume their own feces). For pure cultures of laboratory strains, selective medium supplementation is less critical, but in order to simplify preparation of bacterial growth plates, it will do no harm to always use plates prepared with the indicated supplements.

## BASIC PROTOCOL 1

### CULTURE OF *HELICOBACTER* ORGANISMS ON SOLID MEDIUM

*H. pylori* is a fastidious organism capable of growing only under a narrow set of conditions. It must be cultured on nutrient-rich agars such as Columbia blood agar base supplemented with whole blood from any one of a variety of large mammals such as horse, ox, or sheep. Reduced oxygen tension is crucial; therefore, a closed-container system capable of generating microaerophilic conditions is usually employed. Many laboratories have had success growing *H. pylori* on plates using a tissue culture incubator set at 37°C and either 5 or 10% $CO_2$ with a tray of distilled water set in the bottom of the incubator to maintain humidity. Depending on the isolate, inoculum dose, and freshness and moisture of the plates, growth is generally observed between two and five days. However, some laboratories have

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been unable to achieve *H. pylori* growth using this method. Therefore, the most widely reliable method remains a closed-container system. Typically this consists of an anaerobic jar with a microaerophilic system envelope. It is also necessary to incubate for several days at 37°C before colonies can be easily observed. The different gastric *Helicobacter* species such as *H. pylori, H. felis*, and *H. mustelae* are distinct in colony morphology. *H. felis* is extremely motile and readily forms confluent lawns which, to the untrained eye, can resemble uninoculated plates. Therefore, the eye must be trained to detect *H. felis* growth as a sheen or haze on the plate. *H. pylori* and *H. mustelae* form small, round, translucent colonies which may first be visible at pin-prick size as early as 2 to 4 days, gradually becoming larger.

### Materials

Freshly prepared blood agar plates containing appropriate antibiotic (see Support Protocol 1)

Plates containing viable *Helicobacter* colonies, liquid culture of *Helicobacter* (Basic Protocol 2), or frozen stock of *Helicobacter*

Disposable bacterial spreaders (PGC Scientific), laboratory-made glass spreaders, or inoculating loops

Anaerobic jar(s) with sealable lid(s) (BBL model no. 100 uses one microaerophilic system envelope and holds 12 plates; model 150 uses three GasPak envelopes and holds 36 plates.) Alternatively BD GasPak™ EZ Standard (holds 12–18 plates) or Large (holds 30–33 plates) Incubation Containers or container/microaerophilic systems from other manufacturers can also be used

Microaerophilic system envelopes such as BD GasPak™ EZ Campy paper sachet (Becton Diskinson catalogue #260680) or Oxoid CampyGen™ sachet (Oxoid catalogue CN0025 or CN0035), both of which contain activated carbon and ascorbic acid. These system envelopes have replaced BBL CampyPak Plus Microaerophilic System Envelopes and Oxoid Gas Generating Kits--Campylobacter System (catalogue # BR0056 and BR0060) in many countries. The latter systems, which may still be available in some countries, used palladium catalysts and generated carbon dioxide and hydrogen to deplete oxygen, whereas the newer replacement systems do not use catalysts and generate only carbon dioxide.

1. Inoculate freshly prepared blood agar plates with several colonies from a viable plate using an inoculating loop; alternatively inoculate 20 to 100 µl of thawed material from a frozen stock or liquid culture.

2. Use a bacterial spreader or inoculating loop to spread the material back and forth across the entire plate from top to bottom. Turn the plate 90° and repeat.

3. Cover the plates and place them in anaerobic jar(s).

   When culturing H. pylori and H. mustelae on plates, keep the plates inverted during incubation. For Helicobacter felis, however, do not invert the plates. H. felis is very motile, and the extra moisture that drips down onto the plate when cultured in the noninverted fashion helps it to grow (see Critical Parameters and Troubleshooting and APPENDIX 4A).

   Some laboratories include a water-soaked paper towel in the anaerobic jar to provide a humidified atmosphere. However, adding water to some types of microaerophiloic system envelopse (see step 4) helps accomplish the same purpose. Because of the high moisture content in the anaerobic jars, it is important to disinfect the jars thoroughly between uses; it may be useful to

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include an antifungal and/or selective antibiotic supplement in the medium as well (see Table 8B.1.1).

4. If using a catalyst containing a *microaerophiloic system* envelope, cut the corner off, separate the edges, and place inside the anaerobic jar with the catalyst facing the outside. Add 10 ml water to each envelope, then seal the top of the anaerobic jar. Place the jar in a 37°C incubator or environmental warm room. The newer activated charcoal/ascorbic acid containing sachets do not require water but we have found that including a moist paper towel inside the jar may enhance Helicobacter growth and recovery of organisms from biopsies.

   There are several different types of gas-generating microaerophiloic system envelopes available. Be sure to select the correct one or the Helicobacter organisms may not grow. Note that H. pylori is microaerophilic, not anerobic. Therefore do not select a system designed for anerobes (no or very low oxygen).

   If an incubator is available in which three gases can be controlled, or if custom-blended gas cylinders can be obtained, these may be used in lieu of anaerobic jars and gas generating system envelopes. The incubator should be set to deliver 5% to 7% $O_2$/10% $CO_2$ with the balance $N_2$. If the incubator allows control of two gases plus air, set it at 20% to 35% air (which yields 5% to 7% oxygen), 10% $CO_2$, and the balance $N_2$. Note also that we have had some success using standard $CO_2$ incubators set at 5% to preferably, 10% $CO_2$. Some trial and error may be required to determine the optimum gas mixture for a particular incubator.

5. After 3 to 4 days open the jar and examine the plates for growth. If no growth or only low growth is observed, plates can be returned to the jar with a fresh GasPak for another 2 to 3 days.

   H. pylori and H. mustelae will form distinct pinhead-sized colonies which gradually become larger and easier to identify (Figure 1). Colonies on densely cultured plates may merge into a confluent lawn that displays a distinctive sheen when the plate is viewed at an angle. Thus, if individual H. pylori or H. mustelae colonies are to be counted, timing is important and it may be necessary to examine the plates at two or three different time points. H. felis tends to form confluent sheets within several days of culture. Early examination may allow for enumeration of individual colonies, but these colonies may be very small and difficult to detect.

## SUPPORT PROTOCOL 1

### PREPARATION OF BLOOD AGAR PLATES FOR GROWTH OF *HELICOBACTER* SPECIES

Successful culture of *Helicobacter* organisms requires the use of fresh nutrient blood agar plates. Commercially prepared plates may work, but the freshness of these plates cannot be controlled and they can often be too old or too dry. Additionally, they will lack the appropriate selective antibiotics. Unless one has access to a reliable bacteriology laboratory that can prepare fresh plates to specifications, it is better to prepare them in one's own laboratory. Any one of several nutrient agars recommended for growth of fastidious organisms will work, including Columbia Agar Base, Mueller-Hinton Agar Base, and Blood Agar Base (available from BBL, a trademark of Becton Dickinson). The important thing is that the plates be relatively fresh and moist. When preparing the plates, let the agar harden only until it solidifies (not overnight). If the plates are not to be used immediately, store in sealed plastic sleeves at 4°C for no longer than 2 to 3 weeks.

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### Materials

Nutrient agar base: Columbia Agar Base, Mueller-Hinton Agar Base, or Blood Agar Base (available from BBL; also see *APPENDIX 2C*)

Stock solutions of antibiotics (see Table 8B.1.1)

Defribrinated whole blood (horse, sheep, or ox), sterile (Cleveland Scientific)

1000-ml Erlenmeyer or round-bottom glass flasks or 1000-ml tissue culture bottles

56°C water bath

100-mm sterile polystyrene petri dishes with sleeves

1. Weigh out manufacturer's recommended amount of nutrient agar base for 500 ml medium and add to a flask or bottle containing 500 ml distilled or tap water. Swirl until all the powder is dispersed and freely suspended.

    Settling will occur but it is best not to have any deposits of dry medium when autoclaving.

2. Loosely cover vessels and autoclave under standard conditions for liquid reagents (18 psi/120°C) for 20 min.

3. Remove containers from autoclave and place in a 56°C water bath.

    This allows the medium to cool enough for addition of the blood and antibiotics without denaturing their activity but still being warm enough to prevent the agar from solidifying.

4. Add the appropriate amount of the antibiotics required for the particular *Helicobacter* species (see Table 8B.1.1).

5. Add 35 ml defibrinated whole blood, previously warmed to room temperature.

    Alternatively, "laked" whole blood, which has been frozen and thawed to produce hemolysis, may be used; however, the authors have not found this to be necessary.

6. Remove the flask from the water bath and gently mix until the blood is distributed uniformly. Keep the formation of bubbles to a minimum.

    This is best accomplished by rolling the flask at an angle on the counter top.

7. Immediately dispense the medium into 100-mm petri dishes and remove bubbles by flaming. Stack the warm plates into groups of four or five and allow to cool at room temperature (only until agar has hardened).

    Stacking will allow the agar to cool more slowly and minimize the amount of condensation that forms on the lids (except for the top plate). However, unstacked plates will solidify more quickly.

8. Following solidification of the agar, place plates in a plastic sleeve and store at 4°C until needed.

## BASIC PROTOCOL 2

### CULTURE OF *HELICOBACTER PYLORI* IN LIQUID MEDIUM

*Helicobacter* may also be grown in liquid media formulations. Several strategies can be used, depending on the species. Several nutrient-rich media such as Brain Heart Infusion or *Brucella* broth will support growth when supplemented with 10% heat-inactivated fetal bovine serum (FBS). Reduced oxygen tension can be supplied either through the use of a

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closed container system capable of generating microaerophilic conditions, or, in the case of *H. pylori*, by using a $CO_2$ incubator. Incubation at 37°C is required for growth. Exponential growth or even saturated cultures can be obtained in as little as 24 hr, depending on the inoculating dose.

## Materials

*Brucella* broth or other suitable liquid medium supplemented with 10% (v/v) heat-inactivated FBS

Agar plate containing a heavy growth of viable *H. pylori* (Basic Protocol 1)

Disposable bacterial spreaders (PGC Scientific) or laboratory-made glass spreaders

25-$cm^2$ polystyrene tissue culture flasks

37°C, 5% $CO_2$ incubator

1. Place 1 ml of *Brucella* broth/10% FBS medium on to the center of an 100-mm *H. pylori* plate culture.

2. Using a sterile bacterial spreader, lift *H. pylori* from plate by continuous gentle raking of the surface. Slightly tip plate forward and gather liquid towards the front rim.

3. Transfer 100μl or more of the suspension of *H. pylori* to a 25-$cm^2$ tissue culture flask containing 10 ml of *Brucella* broth/FBS media.

   The recovered volume will be <1 ml as some of the liquid is absorbed by the solid medium. If the entire volume is transferred a saturated culture could result overnight.

4. Attach the cap of the flask so that it is loose enough to allow gas exchange but not loose enough to fall off.

   Alternatively, flasks with vented caps may be used and screwed on tightly.

5. Place flask in a 37°C, 5% $CO_2$ incubator standing upright.

   The authors have noticed that keeping the flask upright results in more reliable growth, possibly due to the increased depth of the medium.

6. Allow culture to grow overnight. Check for *Helicobacter* growth by gently agitating the flask to suspend the bacteria and then placing the flask horizontally on an inverted microscope. *H. pylori* growing in liquid culture tends to form small to large aggregates and after agitation of the flask has stabilized and larger aggregates have settled to the bottom surface of the horizontal flask, it may be useful to focus on these aggregates at low (4× or 10× objective) magnification to find a depth of focus where individual *H. pylori* can be observed. Then with a 20× or preferably a 40× microscope objective look for highly motile bacteria as an indication of a healthy, highly viable culture. Phase objectives may enhance visualization of liquid cultures. (See Supplementary Material, Supp Movie S1 and Supp Movie S2)

7. Passage culture by transferring a small aliquot to another flask containing 10 ml *Brucella* broth/FBS medium.

   Passage before viability begins to decline (OD of 0.2 to 0.3 or lower).

   The volume of the aliquot used for passage is highly variable and must be based on the density of the culture and how soon one would like the new culture to reach saturation. Generally, 100 to 200 μl of a healthy culture will

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generate a saturated culture within 24 to 48 hr. At this point, larger cultures can be started by using 60 ml medium in a 75-cm$^2$ flask or 100 ml in a 150-cm$^2$ flask.

## ALTERNATE PROTOCOL

### CULTURE OF *HELICOBACTER* SPECIES OTHER THAN *H. PYLORI* IN LIQUID MEDIUM

Static liquid cultures able to grow under standard tissue culture incubator conditions have only been described for *H. pylori* (see Basic Protocol 2). Other gastric *Helicobacter* species such as *H. felis* require continuous shaking and a micro aerophilic closed culture systems e.g. the authors have had poor success attempting to grow *H. felis* in liquid culture with agitation in a $CO_2$ incubator; therefore, alternative methods for growth are required. Unavailability of a $CO_2$ incubator or unwillingness of colleagues to allow the use of a tissue culture incubator for growth of bacterial cultures (because of concerns about contamination) may also favor alternative strategies. Most gastric pathogens are readily grown in the same liquid culture medium described in Basic Protocol 2 when placed in a suitable flask within an anaerobic jar on a rotary shaker.

#### Additional Materials (also see Basic Protocol 2)

Automatic pipetting device (e.g., Drummond Pipet-Aid or, for volumes  1 ml, Gilson Pipetman or equivalent automatic pipettor)

5-ml culture tubes

500-ml Erlenmeyer or tissue culture bottles that fit inside BBL model no. 100 anaerobic jar

Anaerobic jar(s) with sealable lid(s) (BBL model no. 100 or equivalent)

Microaerophilic System Envelopes (see Basic Protocol 1)

37°C rotary shaker with 2-liter brackets to accommodate a BBL model no. 100 anaerobic jar

Additional reagents and equipment for quantification of *Helicobacter* (see Basic Protocol 2; see Basic Protocol 3 for *H. felis*) and examining wet mount of *Helicobacter* (Support Protocol 4)

1. Place 1 ml *Brucella* broth/10% FBS medium on to the center of an *H. pylori* plate culture.
2. Using a sterile bacterial spreader, lift *H. felis* from plate by continuous gentle raking of the surface. Slightly tip plate forward and gather liquid towards the front rim.
3. Transfer harvested bacteria into a 5-ml sterile tube using an automatic pipetting device.
4. Place an aliquot equivalent to the bacteria harvested from one-half of a plate (0.5 ml) into a sterile 500-ml Erlenmeyer flask containing 100 ml Brucella medium/ FBS.

   It is also possible to passage a small volume of an existing, actively growing liquid culture. The inoculum will vary between 1 and 10 ml depending on the viability of the culture and the speed at which one wishes new growth to occur.

5. Place the flask containing the inoculated medium within an anaerobic jar.

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6. Cut the corner off of the envelope if using gas generating system envelopes with catalyst, separate the edges, and place inside the anaerobic jar with the catalyst facing the outside. Add 10 ml water to each envelope, then seal the top of the anaerobic jar. If using non-catalyst sachets, remove the sachet from the foil wrap and insert into the jar and seal the top of the jar.
7. Place the sealed jar in a 2-liter flask bracket on an orbital shaker at 37°C.
8. Incubate with shaking at 100–125 rpm for 24 to 48 hr.
9. At the end of this incubation, or earlier, remove an aliquot of the culture and determine optical density (see Support Protocol 2; see Support Protocol 3 for *H. felis*).

   An optical density of 0.1 at 550 nm should yield ~$10^7$ cfu/ml, but this should be verified by means of one's own growth curve (see Support Protocol 2).
10. Prepare a wet mount (see Support Protocol 4) and examine for motility and absence of contamination with other bacteria.

## SUPPORT PROTOCOL 2

### QUANTIFICATION OF *HELICOBACTER* ORGANISMS BY CULTURE

Both *H. pylori* and *H. mustelae* will form individual colonies when grown on a blood-containing agar base. Thus, serial dilution and counting of colonies can be use to quantify both *H. pylori* and *H. mustelae*. However, for speed and convenience it may be more efficient to prepare a growth curve so that the concentration of bacteria can be determined simply by measuring optical density while growing as a liquid culture.

The following method uses *H. pylori* as an example; however, other *Helicobacter* species can also be quantified using this procedure, with the exception of *H. felis*, which should be treated as described in Support Protocol 3.

#### Materials

*Brucella* broth or other suitable liquid medium supplemented with 10% (v/v) heat-inactivated FBS

Actively growing liquid culture of *H. pylori* (see Basic Protocol 2 or Alternate Protocol)

Freshly prepared blood agar plates containing appropriate antibiotic (see Support Protocol 1)

25-$cm^2$ tissue culture flask

37°C, 5% $CO_2$ incubator

Spectrophotometer with visible wavelength spectrum, preferably with adapter for microcuvette

Cuvette or microcuvette

Sterile tubes for preparing dilutions

Disposable bacterial spreaders (PGC Scientific) or laboratory-made glass spreaders

Additional reagents and equipment for microaerophilic plate culture of *Helicobacter* (Basic Protocol 1)

1. **1.** Place 10 ml *Brucella* broth/5% FBS media in a 25-$cm^2$ tissue culture flask and inoculate with 100 μl of a log-phase culture of *H. pylori*. Place in a 37°C, 5% $CO_2$ incubator.
2. **2.** Several times each day, resuspend the bacteria uniformly by swirling the flask, remove a 100-μl aliquot, and transfer to a cuvette or microcuvette for the spectrophotometer.

   Depending on the viability of the starting culture, it may take 1 to 3 days, or even longer, to complete the growth curve.
3. **3.** Determine the $OD_{550}$ of the culture using *Brucella* broth/10% FBS as a blank.
4. **4.** Using sterile tubes, prepare serial 10-fold dilutions of the aliquot from $1{:}10^2$ to $1{:}10^5$.
5. **5.** Inoculate 10 μl of each dilution onto a blood agar plate supplemented with antibiotics and spread uniformly using a bacterial spreader until no liquid remains on the plate. Mark each plate or fraction of plate used with the time point and dilution.

   To reduce the number of plates required, each plate can be divided into half or even thirds using a marker on the bottom of the plate and distributing each 10-μl inoculum only within the marked boundaries.
6. **6.** Cover plates and place in anaerobic jars with GasPaks as described in Basic Protocol 1.
7. **7.** Repeat steps 2 to 6 with the liquid cultures until $OD_{550}$ approaches 0.5.
8. **8.** Evaluate plates at 4 to 5 days and enumerate colonies, back-calculating for dilution and volume to determine the number of colony forming units (cfu) per milliliter.

   For example, if 10 μl of a 1:1000 dilution yields 25 colonies, then there are $100 \times 1000 \times 25$ cfu = $2.5 \times 10^6$ cfu per milliliter of the original sample.
9. **9.** Plot $OD_{550}$ versus cfu to obtain the growth curve.

   As noted above the authors have found that an $OD_{550}$ of 0.1 generally yields ~$10^7$ cfu in their laboratories. However, the specific conditions in another laboratory could be quite different, so it is best to complete one's own growth curve.
10. **10.** Repeat this procedure at least twice and average the results in order to obtain a reasonably accurate growth curve.

# SUPPORT PROTOCOL 3

## QUANTIFICATION OF *HELICOBACTER FELIS* BY CULTURE

*H. felis* will not reliably form individual colonies, and even when individual colonies are observed with *H. felis*, these colonies may under-represent the number of viable organisms present. To attempt an estimation of the number of "cfu" of *H. felis* in stock cultures, the authors have used two approaches. The first is to use an *H. pylori* growth curve as a surrogate for an *H. felis* growth curve. Therefore, if the *H. pylori* growth curve indicates that an $OD_{550}$ of 0.1 equals $10^7$ cfu of *H. pylori*, a reasonable extrapolation would be that an $OD_{550}$ for a culture of *H. felis* would also represent ~$10^7$ cfu of *H. felis*. The second approach is to prepare a subconfluent culture of *H. felis* and make serial dilutions of this culture into *Brucella* broth. Pipet an aliquot of appropriately diluted culture into a hemacytometer (*APPENDIX 4A*) and quickly count the number of motile organisms at



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400× total magnification. This is difficult, as a high-power microscope objective must be used and *H. felis* bacteria are highly motile and can move into and out of the hemacytometer grid squares. The authors have found that these two methods give numbers which are in reasonable agreement with each other.

## SUPPORT PROTOCOL 4

### QUANTIFICATION OF *HELICOBACTER* BY QUANTITATIVE PCR

The quantification of *H. pylori* and *H. felis* in gastric biopsies can be achieved by quantitative PCR using a standard curve consisting of defined concentrations of chromosomal DNA from the respective bacteria. These procedures rely on the collection of suitable biopsies that include portions of the stomach most likely to be colonized with Helicobacter organisms and the efficient isolation of total DNA from the biopsy. This assay is suitable to detect fewer than 50 organisms but unlike quantification by culture, this assay does not differentiate between viable and nonviable bacteria. The protocol outlined below for *H. pylori* quantification based on detection of the *ureC* gene is a modification of the procedure developed by He at al. (He et *al.* 2002). The protocol and primers specified for *H. felis* 16S RNA were reported by Kong et al (Kong et al. 1996). Other investigators have published PCR based assays using primers for other Helicobacter genes, but the authors have successfully utilized the PCR assays included here. Like all PCR protocols, extreme caution should be used to minimize the likelihood of cross contamination of samples through the use of filter tips, clean space, and dedicated reagents.

#### Materials

DNA isolation kit

Purified chromosomal DNA from *H. pylori* or *H. felis*

Real time thermocycler

Multiwell PCR assay plates

*H. pylori ureC* primers (Forward 5’ – TTATCGGTAAAGACACCAGAAA – 3’; Reverse 5’ – ATCACAGCGCATGTCTTC – 3’)

or

*H. felis* 16S rRNA primers (Forward 5’ - ATGACATGCCCTTTAGTTTGGGATAGCCA-3’; Reverse 5’- CGTTCACCCTCTCAGGCCGGATACC-3’)

SYBR Green mastermix

1. Determine the concentration of Helicobacter DNA isolated from a pure culture of *H. pylori* bacteria using a DNA isolation kit by measuring the absorbance at 260 nm.
2. Calculate the copy number concentration as the number of Helicobacter chromosomes/microliter [(concentration of DNA ng/μl)/(mass of Helicobacter genome)]. The mass of the Helicobacter genome is approximately 1.65 Mb which translates to approximately $1.78 \times 10^{-6}$ ng.
3. Prepare a 10-fold dilution series of the chromosomal Helicobacter DNA with the highest concentration in the order of $10^7$ copies and the lowest in the order of $10^1$ copies.
4. Isolate total DNA from gastric biopsy tissue using a commercial DNA isolation kit.



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It is important to establish the weight of the tissue biopsy prior to isolating the total DNA. Tubes to be used to hold tissue biopsies should be pre-weighed, and then weighed a second time once biopsy has been placed inside. The weight of the tube can then be subtracted from the total to establish the wet weight of the biopsy.

It is also very important to take steps to optimize the recovery of the Helicobacter DNA from the tissue biopsy as the DNA isolation procedure will isolate tissue DNA as well. For most kits, the protocol to be followed will be specific for isolating total DNA from the tissue. Modifications should be made to increase the recovery of DNA from the bacteria such as an additional step at 95°C for 10 minutes following Proteinase K digestion. Consult the manufacturer for modifications and recommendation. The DNeasy kit from Qiagen makes specific recommendations for isolating the bacterial DNA from tissue samples.

Lastly, do not use tissue pieces that are larger in mass than recommended for a DNA isolation reaction. This will result in reduced recovery and DNA purity.

5. Prepare assay plate for quantitative PCR. Each sample should be prepared in triplicate using 3 μl extracted sample DNA plus 5 mM of each of the relevant primers, and the SYBR green master mix. Bring the total volume up to the manufacturer's recommended volume with water. In the same plate, set up the dilution series of standard chromosomal DNA prepared in step 3 above in triplicate with the relevant primers and SYBR Green. Remember to include a set of three wells with no template DNA as a control.

6. For quantification of *H. pylori* using ureC specific primers, perform the reaction with a preliminary denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 55°C for 10 sec, and primer extension at 72°C for 20 sec.

   For quantification of H. felis using 16S RNA specific primers, perform a two step reaction consisting of one cycle of 50°C for 2 min followed by 95°C for 10 min, then 40 cycles of denaturation at 95°C for 10 sec and annealing/ extension at 60°C for 60 sec.

7. Program the thermocylcer to calculate the mean number of copies of DNA in each triplicate sample based on the standard curve.

8. Determine the bacterial load in the tissue biopsy by extrapolating the total number of copies in the total volume isolated from the DNA isolation procedure based on the number of copies in the 3 μl used for the reaction. Divide this number by the tissue mass to obtain number of copies per mg tissue.

## BASIC PROTOCOL 3

### CULTURE OF *HELICOBACTER* ORGANISMS IN AGAR STABS

The continuous culture of Helicobacter organisms in vitro utilizes a lot of resources in the form of blood products, media, and gas pak systems because *H. pylori* generally cannot be stored for prolonged periods of time without using frozen storage systems. Frequent passage, occurring every several days or at least once per week, also increases the likelihood of introducing airborne molds and other contaminants to the cultures. Some of these problems can be circumvented by preparing agar stabs for the long term storage of *H. pylori* or *H. felis* at 37°C (Xu et al. 2010. Visible growth can be observed in these agar stab cultures within several days of inoculation and will remain viable for up to a month in the

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case of *H. felis*, or for at least several months in the case of *H. pylori*. It has also been reported that slight modifications of this method can be used as an alternative to sending frozen cultures on dry ice as a method for shipping *H. pylori* to other investigators (Reddy et al., 2011).

### Materials

Active *Helicobacter* culture growing on Columbia Blood Agar or acceptable alternative

Brucella broth or Brain heart Infusion media containing 0.6% agar _

12 × 75 mm 5 ml round bottom polypropylene tubes with snap caps (BD Bioscience)

Disposable 1 ul inoculating loops

1. Autoclave media under standard conditions for liquid reagents (18 psi/120°C) for 20 min.
2. Dispense media using a pipet under sterile conditions, four ml per tube. Place caps loosely on tubes and let solidify overnight.
3. Collect a heavy inoculum of bacteria from the Columbia Blood Agar plate using a disposable transfer loop.
4. Stab loop into the tubes about two thirds the depth of the agar three to four times.
5. Place caps loosely on tubes.
6. Place tubes in 37°C tissue culture incubator at 5 or 10% $CO_2$ with a water pan placed in the bottom of the incubator to maintain humidity.
7. Check for "cloudy tracks" in the agar indicative of growing bacteria.

   *It may be necessary to hold the tubes overhead with the ceiling forming the background and looking at the tubes at different angles to observe the cloudiness that forms as a result of increasing bacterial density. This cloudiness may not be apparent until three or four days.* (Figure 2)
8. Snap caps onto tubes tightly and continue to store in tissue culture incubator or at room temperature.

## BASIC PROTOCOL 4

### PREPARING CLINICAL BIOPSIES FOR CULTURE OF *HELICOBACTER PYLORI*

Gastric pinch biopsy material is routinely taken during endoscopy from subjects presenting with symptoms of active gastritis or peptic ulcer disease. In addition, in animal models, confirmation and quantitation of infection is often an end point. Along with identification of *H. pylori* in histologic tissue sections, culture of viable *H. pylori* remains the definitive test for a positive diagnosis of *H. pylori* infection. Additionally, the tremendous genetic and phenotypic diversity between *H. pylori* isolates provides incentives for continuing to isolate novel clinical isolates for further experimental analysis in the laboratory. *H. pylori* can be recovered from the gastric biopsy material of infected subjects, or experimentally infected animals, by homogenizing the tissue and inoculating nutrient blood agar plates. The use of a urease test broth (see Support Protocol 4) to store or transport the biopsy can provide preliminary evidence of the presence of *H. pylori* if the indicator dye turns bright red within 24 hr of retrieval.

Usually, several pinch biopsies can be obtained from each subject during endoscopy. One or more biopsies are placed into a single tube for culture. In addition to culture, it is generally useful to send additional biopsies to a histology lab for embedding, sectioning, and staining.

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These biopsies should be collected into 10% buffered formalin. If sufficient biopsies are available, it may also be useful to deposit one or more into a freezing vial and then immerse into liquid nitrogen and store at −80°C or lower, e.g., for RNA isolation.

### Materials

Gastric pinch biopsy material

Transport medium (see recipe) in sterile tubes

*Brucella* broth or other suitable liquid medium supplemented with 10% (v/v) FBS

Freshly prepared blood agar plates containing appropriate antibiotic (see Support Protocol 1)

Sterile forceps

Sterile disposable pellet pestles and 1.5-ml microcentrifuge tubes (Kontes, cat no. 749520)

Disposable bacterial spreaders (PGC Scientific) or laboratory-made glass spreaders

Additional reagents and equipment for microaerophilic plate culture of *Helicobacter* (Basic Protocol 1)

1. Retrieve gastric biopsy material from clinical laboratory in transport medium.

   Biopsies should be obtained for culture as soon as possible after endoscopy, although the authors have had some success in culturing from biopsies stored overnight at room temperature in transport medium.

2. Using sterile forceps, remove biopsy pieces from the transport medium tubes and place in sterile 1.5-ml microcentrifuge tube (provided along with a disposable pellet pestle) with 0.1 ml *Brucella* broth/10% FBS.

3. Grind the tissue using the sterile disposable pellet pestle and add an additional 0.1 ml *Brucella* broth/FBS.

4. Inoculate 10 μl of the homogenate onto a blood agar plate with the appropriate selective antibiotics and spread with inoculating loop or spreader.

5. Cover plates, place in anaerobic jars with microaerophilic system envelopes, and culture as described in Basic Protocol 1, steps 3 to 5.

## SUPPORT PROTOCOL 5

### CONFIRMATION OF *HELICOBACTER PYLORI* GROWTH

Although the use of selective antibiotics will greatly reduce the growth/isolation of non-*Helicobacter* organisms from animal stomachs, it is still necessary to be sure that the organisms recovered are in fact a *Helicobacter* species. While nucleic acid fingerprinting or PCR procedures can be employed for this, there are also simple morphologic and biochemical tests which can be used to screen suspected *Helicobacter* growth on plates. This is done by using an inoculating loop to pick material from a plate with visible growth and then examining the morphology of the organisms by phase-contrast microscopy of a wet mount and/or of Gram-stained organisms (*APPENDIX 3C*). Additional confirmatory procedures include testing the bacteria for urease, catalase and oxidase activity; these techniques are also described below.

All of the gastric *Helicobacter* species produce large amounts of the enzyme urease, which can cleave urea into carbon dioxide and ammonia. The ammonia is thought to buffer the

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acidity of the stomach and thus aid the bacteria in its colonization. It is possible, particularly in experimental animals, for other urease-positive micro-organisms (e.g., *Proteus* species) to colonize the stomach. Thus, the combination of colony morphology, urease test, oxidase test, catalase test, and spiral morphology/motility in a wet mount are used to confirm the identity of *H. pylori*. As noted above, nucleic acid fingerprinting and PCR techniques can be used for further verification.

**Wet mount/morphology—**Viable *Helicobacter* species will be highly motile, and if enough bacteria are placed on the slide they can be easily located by phase-contrast microscopy at 400× to 1000× total magnification. *H. felis* is relatively long and possesses a distinct corkscrew morphology with tight spirals. *H. pylori* and *H. mustelae* are shorter with less distinct spirals. In general, they have a comma- or gull wing–like shape. In a healthy culture, the bacteria will appear to be “swimming” about quite rapidly. An overgrown culture may yield an optical density in the desired range, but observation of low or no mobility will indicate lack of viability. Thus it is important to always check cultures for morphology and motility.

**Urease test—**Place a loopful of material into 0.3 ml Stuarts urease test broth (see recipe). The broth should turn pink within minutes.

**Catalase test—**Place a loopful of material into 0.2 to 0.5 ml of 3% $H_2O_2$. Viable *Helicobacter* will rapidly form bubbles. This can be done in a 96 well microtiter plate if multiple samples are to be tested.

**Oxidase test—**Either place a loopful of material into a drop of distilled water on a Bacto Differentiation Disk Oxidase (BD Biosciences, cat. no. 1633) or place a disk over a colony or area of growth on a plate with a drop of distilled water. A pink or maroon color within 10 to 20 min, eventually changing to almost black, indicates a positive test.

## REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see *APPENDIX 2A*; for suppliers, see *SUPPLIERS APPENDIX*.

### Stuart’s urease test broth

0.1 g yeast extract

0.091 g $KH_2PO_4$

0.095 g $Na_2HPO_4$

20.0 g urea

0.010 g phenol red

1 liter distilled $H_2O$

Adjust pH to 6.9 using a small quantity of HCl

Note that this recipe has 100-fold less buffering capacity than some commercially available urease test broths. If the test broth is used with a higher buffer concentration, it will be less sensitive and will give many false negatives. The authors usually make only 500 ml at a time and then use part of that as transport medium (see recipe).

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### Transport medium for Helicobacter

To Stuart's urease test broth (see recipe), add 20 g glucose or sucrose per 100 ml. Filter sterilize and store up to several months at 4°C.

## COMMENTARY

### Background Information

*Helicobacter pylori* is a Gram-negative microaerophilic spiral bacterium which predominantly inhabits the antral region of the human stomach (Marshall and Warren, 1984; Suerbaum and Michetti, 2002). While *H. pylori* can be found in the body or fundus of the stomach or in the gastric metaplasia of the duodenum, it seems well adapted only to gastric tissues. It is not found, for instance, in normal small or large intestine or in the esophagus. The organism resides within the mucus layer of both surface and glandular epithelium. This ecologic niche serves to protect the organism from the harsh environmental conditions of the stomach and from host effector mechanisms. However, despite the fact that host responses are ineffective in clearing the organism, *H. pylori* induces a state of active/chronic gastric inflammation (involving both neutrophils and lymphocytes) in all infected individuals. While there is no overt disease in most infected people, a subset of infected individuals may develop peptic ulcers, gastric cancer, or gastric mucosa–associated lymphatic tissue (MALT) lymphoma. The outcome of infection is determined by a complex interplay between bacterial virulence factors and the host response. Individuals with high gastric acid output who are infected with virulent *H. pylori* strains may be predisposed towards antral-predominant infections and gastritis leading to peptic ulcer disease, while those individuals with lower gastric acid are prone to infection of both the antrum and body of the stomach, and to pangastritis, atrophy of glandular and parietal cells in the body/fundus of the stomach, and gastric adenocarcinoma.

Assessment of the *H. pylori* genome has revealed a great deal of diversity between isolates (Jiang et al., 1996; Tomb et al., 1997; Suerbaum, 2000; Kawai et al., 2011). Thus, although the complete genomes of twenty distinct isolates have been published, a hypervariable region of the chromosome has been identified that can house up to 7% of the total number of genes (Alm et al., 1999). Many of these genes are specific to individual isolates. This can make complete characterization of the *H. pylori* phenotype difficult. Additionally, although plasmids have been identified in *H. pylori* strains, few stable *Helicobacter* plasmids suitable for cloning and expression techniques have been described or developed (Kleanthous et al., 1991; Stanley et al., 1992; Bereswill et al., 2005). Introduction of genes directly into the chromosome can be accomplished via natural transformation or electroporation with *E. coli* cloning vectors, but the transformation efficiency of *H. pylori* strains varies dramatically; therefore, specific isolates must be identified that facilitate genetic manipulation (Tsuda et al., 1993).

The procedures in this unit describe how to grow *Helicobacter* organisms using plates, agar stabs, or liquid cultures, how to quantify *Helicobacter* species, and how to recover *H. pylori* from clinical biopsies. *Helicobacter* species tend to grow well on media previously established for other enteric fastidious organisms (e.g., see *APPENDIX 2C*), although the addition of animal blood or sera is often required. The growth of pure cultures of *Helicobacter* species has facilitated the study of *Helicobacter* genetics, microbiology, virulence, and immunology. Since *Helicobacter* species are associated with a growing number of gastric and extragastric pathologies, the ability to grow *Helicobacter* species successfully can be an important skill.

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## Critical Parameters and Troubleshooting

*H. pylori* and other *Helicobacter* species are considered fastidious organisms which can be difficult to isolate and propagate. Both temperature and oxygen tension are critical parameters for optimal growth. If inoculation of plates from a frozen stock is unsuccessful, the condition of the anaerobic jar and the integrity of the seal when assembled should be tested. However, even if no problems can be identified, it is worthwhile to try again, since establishing the initial culture can be difficult (see Anticipated Results). Similarly, it is not uncommon for established cultures to lose viability and fail to grow when passaged. The jar seal should be checked to make sure that the proper oxygen tension is being obtained and test strips to verify that oxygen is being properly depleted can be obtained from the manufacturers. The freshness/moisture of the plates being used to passage the bacteria should also be assessed. The authors have found that the freshness of the horse or other animal blood used to prepare these plates is also important.

Properly acting gas generating systems in these closed-container methods are crucial to their success. Several options have been available to choose from in the past, including systems which used catalysts and generated both hydrogen and carbon dioxide. There are now more limited choices available with BD GasPak™ EZ Campy (Becton Dickinson) and CampyGen™ envelopes (Oxoid), both of which do not use catalysts and generate only $CO_2$ being the most readily available. The authors have used both of these systems to grow *H. pylori* and *H. felis*. These envelopes are easy to use and simply require the user to remove the sachet from within a sealed enveloped and place in the jar with the agar plates immediately prior to sealing the lid. Sealing the jar quickly after adding the sachets is very important, as these systems begin to generate $CO_2$ very quickly once exposed to air. The performance of these packets for *Helicobacter* growth can be made more reliable by placing a damp paper towel in the bottom of the jar before placing the bacterial plates and the gas generating sachets inside. Given the difficulties associated with growing these fastidious organisms, if no growth is observed after five to seven days, it may be worth repeating the procedure and perhaps including test strips to verify $CO_2$ generation/$O_2$ depletion. Once cultures have been established from biopsies or frozen cultures using sealed jars and gas generating systems, the authors have found that standard $CO_2$ incubators set at 5–10% $CO_2$ can be used to maintain plate cultures, liquid cultures, and agar stabs of *H. pylori*. For plate cultures of *H. pylori,* growth tends to be somewhat slower in $CO_2$ incubators than in sealed jars and except for agar stabs, the authors have not had good success growing *H. felis* in $CO_2$ incubators.

Although bacterial contamination of pure cultures is rare when proper technique is utilized, the presence of mold can be more problematic. Generally, mold contamination occurs under two circumstances. First, plates often get contaminated when they are examined for growth and then placed back in the anaerobic jars for further growth. This is in large part due to removal of the plate lid to get a better look at the colony morphology. The second source of mold contamination is often due to the collection of water on the lids of inverted plates. When the water collects between the edge of the plate and lid, it effectively forms a bridge to the outside of the plate. If mold spores are present inside the jar, they may get inside the plate by this water route. A solution is to maintain the plates right side up, but the collection of water droplets on the agar itself can compromise the ability of the bacteria to grow as isolated colonies, and, if plates are being grown for quantitative counts, reliable numbers may be difficult to obtain. Disinfecting the bell jars between uses can also help to reduce mold growth, as can the inclusion of an anti-fungal (amphotericin B) in the growth medium.

If cultures (either plate or liquid) overgrow, the bacteria may die or become coccoid in nature. Although coccoid organisms may not technically be dead, their viability can be difficult to determine. Overgrown, dying, or coccoid cultures will yield a measurable optical

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density; however, this optical density will not be a true reflection of number of fully viable cfu of *Helicobacter* organisms. Thus, examination of liquid bacterial cultures growing in tissue culture flasks or by wet mount of plate cultures for active motility and expected morphology is a critical parameter. (See video clips associated with Basic Protocol 2 and wet mounts in Support Protocol 5). When using a growth curve and the optical density to estimate the number of organisms present, it is critical to be on the "up side" of the growth curve rather than on the "down side" or plateau. *Helicobacter* organisms grow much more slowly than many other bacteria, and it is important to check their progress periodically to avoid waiting too long before passaging them or using them for in vitro analysis.

Except for disease conditions characterized by low stomach acidity, where gastric bacterial overgrowth by many species can occur, *Helicobacter* species are usually the only bacteria that can successfully colonize the human stomach. In coprophagic animal species such as mice, however, numerous bacterial species can routinely be cultured from stomach tissues. Thus, recovering clinical or experimental *H. pylori* isolates from gastric biopsies can be associated with contamination by other bacterial species. Normally, few bacteria survive in the gastric mucosa, but when contaminants are present, *Proteus vulgaris* may be suspected. It tends to be resistant to the common *H. pylori* selective media. This is further complicated by the fact that *Proteus* is one of the few enteric bacteria that also produces a urease enzyme, often leading to false-positive urease tests. *Proteus* also tends to grow quickly and forms large sheets, effectively covering any *H. pylori* that might be present as small isolated colonies. Therefore, it is important to plate many dilutions of clinical biopsy homogenates in the hopes that *H. pylori* can be isolated from the background of *Proteus* growth. The combined results of colony morphology observation and the urease, catalase, and oxidase tests, as well as the distinctive morphology (spiral or gull-wing shape) and high motility of *Helicobacter* organisms observed in a wet mount is usually sufficient to distinguish *Helicobacter* species from *Proteus* species.

## Anticipated Results

Establishing viable cultures of *H. pylori* and other *Helicobacter* species may take several attempts, especially when starting from a frozen stock. However, if multiple plates are inoculated or repeated attempts are made, viable cultures can be obtained as long as the anaerobic jars are properly sealed. Performing subsequent passages from a viable culture is generally much more reliable and the setup is easy to maintain. One can expect to observe fairly large and healthy colonies every 3 to 4 days once these cultures are growing. Likewise, once liquid cultures are established, one should expect to passage cultures every 1 to 2 days. Even if microscopic examination reveals dense growth but minimal motile organisms, these cultures may still be viable and suitable for inoculation into fresh media.

Recovering viable *H. pylori* from clinical gastric biopsies can be challenging. It is common for laboratories that perform this function on a regular basis to fail to isolate *H. pylori* even when the rapid urease test is positive. The site of biopsy source, freshness of the biopsy, antibiotic treatment of the subject, and bacterial density within the biopsy can all affect the success of isolating *H. pylori* from human tissue.

## Time Considerations

The time necessary to culture *Helicobacter* species on solid media will vary, depending on whether or not blood agar plates have been prepared in advance. It will also vary with the number of plates to be inoculated. In general, plates can be prepared and ready for culturing in several hours. Preparation of the medium and autoclaving takes less than an hour. The two most time-consuming steps are allowing the temperature of the media to cool to 56°C, and allowing the medium to gel adequately following pouring of the plates. The whole

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procedure can be accomplished in one morning or afternoon. The actual inoculation of plates and preparation of the jars for incubation take only a few minutes.

The number of bacteria in a stock culture can be approximated in a few minutes by measuring the $OD_{550}$ and referring to a growth curve. Rigorous determination of cfu takes longer, but by using the OD and a growth curve, one or two dilutions can be picked for inoculation onto plates, which takes only a few minutes. The plates must then be incubated for several days, but actual counting of colonies after they appear also takes only a few minutes. Homogenization of tissue biopsies and inoculation of the relevant plates will take a few minutes per biopsy. Counting colonies on plates should only require 1 or 2 min per plate unless a very large number of colonies are present. Likewise, testing the cultures for urease, catalase, and oxidase can be performed in a few minutes, provided that the necessary reagents are prepared ahead of time.

Liquid media can be prepared more quickly than solid media because there is no need to pour plates and allow them to gel. It should only take 2 to 3 hr to prepare, sterilize, and cool the medium (to anywhere between 37°C and room temperature) and much of this time is spent waiting, so that other tasks can be performed concurrently. The medium (without serum or antibiotics) can be prepared in advance and stored for at least 1 month. Adding stock solutions of antibiotics and serum and inoculation of the media with *Helicobacter* organisms takes only a few minutes. Preparation of stock antibiotic solutions can be done in 30 min, if necessary.

Construction of a growth curve for liquid cultures requires 1 to 2 weeks, but a large part of the time is spent waiting for the cultures to grow. The actual hands-on time is much shorter, and once the growth curve is prepared under standardized conditions it need not be repeated unless laboratory conditions change dramatically. The first step in a growth curve is to inoculate a liquid culture. This takes only a few minutes. Then, at regular intervals (from every 30 min to several times per day depending on the rate of growth), the optical density must be determined for an aliquot of the liquid culture, which then also needs to be serially diluted and inoculated onto a fresh series of plates. This procedure may take 1 to 2 hr per day. The fresh plates then need to be incubated for several days until individual colonies are visible. If no colonies are yet visible, the plates are returned to the incubator. When colonies become visible, they must be counted. Simply checking the plates takes only a few minutes, but counting colonies can take 1 to 2 hr depending on how many plates were incubated at the same time. Because the whole procedure will have to be repeated to obtain a reasonable average, it can stretch over perhaps 2 weeks, with actual hands-on time consuming 8 to 16 hr; even more time may be required if the culture plates have not been prepared in advance. Since this is a time-consuming and labor-intensive process, it pays to take great care in doing it and to think twice about altering culture conditions in a way that would require constructing a new growth curve. Note also that, at some time point, the optical density of the growth curve may continue to rise, but that the number of viable cfu will plateau and then decrease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Sutton P, Mitchell H. Helicobacter pylori in the 21st Century, Advances in Molecular and Cellular Microbiology V 17. 2010Wallingford, Oxfordshire, United KingdomCAB International *This monograph contains a collection of contemporary reviews on the bacteriology, pathogenesis,, epidemiology and host response to H. pylori infections.*

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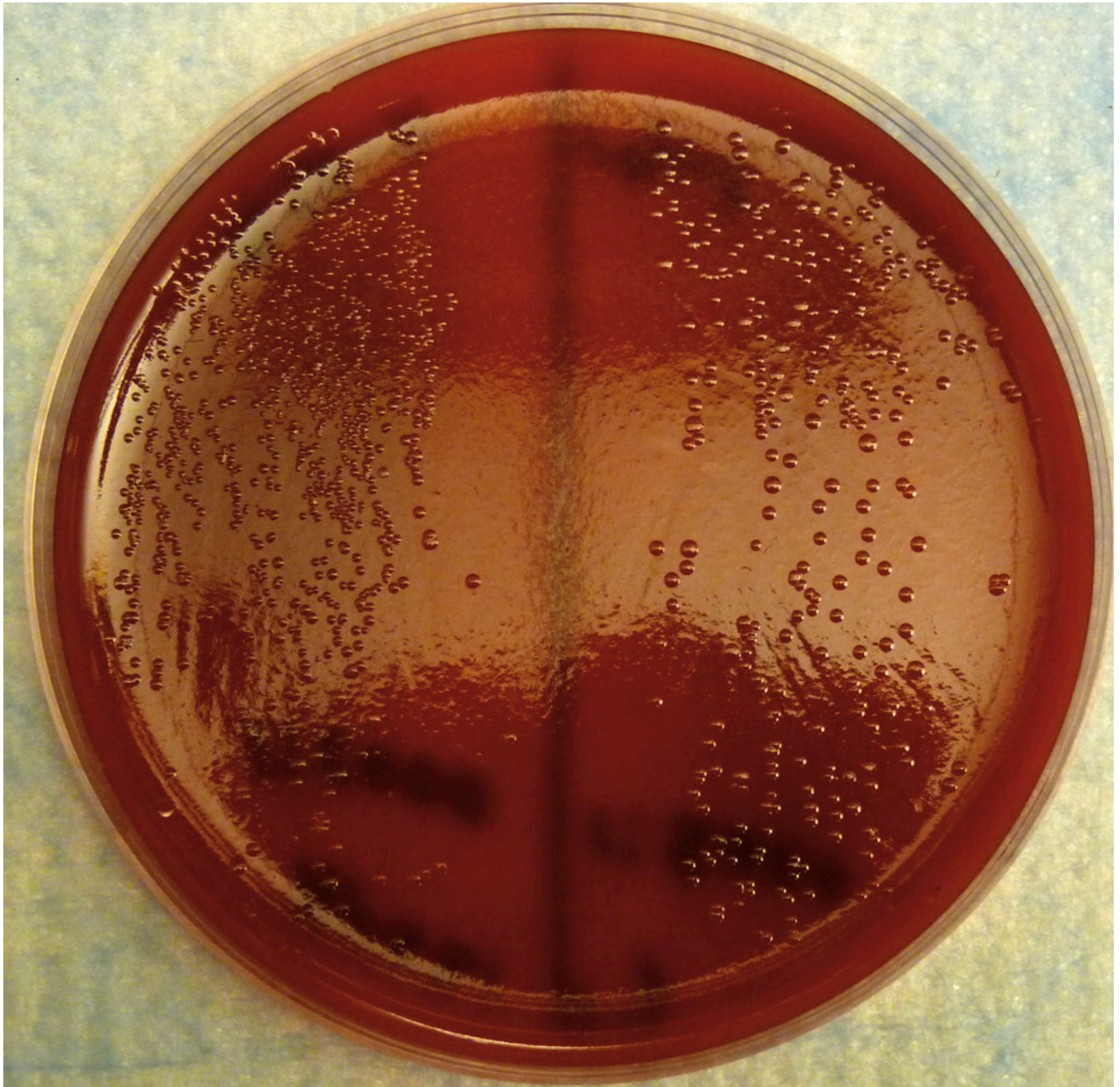

**Figure 1. Morphology of H. pylori Colonies**

*H. pylori* was inoculated onto a blood agar plate 6 days previously. The round translucent colonies illustrate the characteristic appearance of *H. pylori* grown on plates. The distinct colonies on the right half of the plate resulted from diluting the innoculum used on the left side of the plate 3-fold. The colonies on the left side are thus more numerous, and also smaller probably as a result of nutrient depletion. With an even more concentrated innoculum, the individual colonies would merge into a confluent translucent mat, best observed by tilting the plate at an angle to the incident light.

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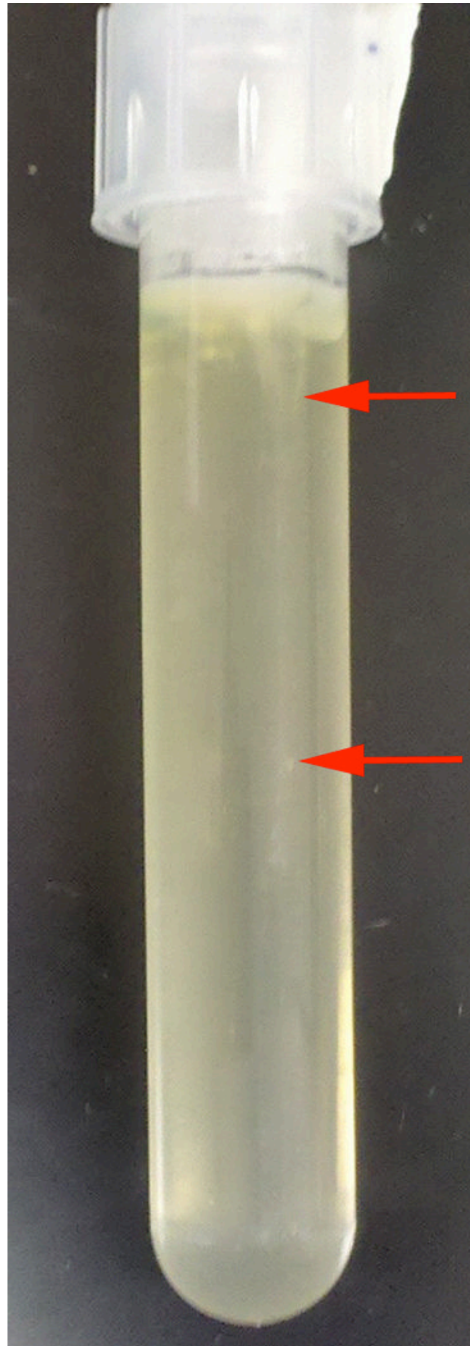

**Figure 2. Agar stab inoculated with H. pylori appearance**

*H. pylori* was inoculated into an agar stab approximately 10 days previously. The two red arrows highlight a "cloudy track" along which *H. pylori* is growing in the agar and from which viable *H. pylori* can be recovered and passaged onto blood agar plates or a liquid culture.

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**Table 8B.1.1**

Supplements for Selective Media

| **Antibiotic** | ***H. pylori*** | ***H. pylori* GSSA**[a] | ***H. felis*** | ***H. mustelae*** |
|---|---|---|---|---|
| Amphotericin B | 2.5 μg/ml | 2.5 μg/ml | 2.5 μg/ml | 2.5 μg/ml |
| Bacitracin | | 20 μg/ml | | |
| Cefsulodin | 16 μg/ml | | | 16 μg/ml |
| Nalidixic acid | | 10.7 μg/ml | | |
| Polymyxin B | | 3.3 μg/ml | 0.125 μg/ml | |
| Trimethoprim | 20 μg/ml | | 20 μg/ml | 20 μg/ml |
| Vancomycin | 6 μg/ml | 10 μg/ml | 6 μg/ml | 6 μg/ml |

[a]GSSA, Glaxo selective supplement A.

[b]For *H. mustelae*, 30 μg/ml cephaloxathin may be substituted for cefsulodin. *H. mustelae* is the only one of these three *Helicobacter* species resistant to cephaloxathin.