PROVIDENCE
Sacred Heart
Medical Center &
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Helicobacter pylori Culture and Antimicrobial Susceptibility Testing Procedure

Department of Microbiology

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1.0 Principle

While there are several noninvasive tests available for the diagnosis of *Helicobacter pylori* infection, a tissue biopsy for culture may be useful for patients with infections that fail to respond to therapy. Gastric biopsy isolates of *H. pylori* should be tested against the antibiotics commonly used in eradication therapy, in particular clarithromycin, as resistance in vitro is clinically relevant. The Clinical and Laboratory Standards Institute (CLSI) has recommended the agar dilution

method for testing susceptibility to clarithromycin. In this method, Mueller-Hinton agar base with 5% aged sheep blood is incubated for 72 h at 35°C, with an MIC breakpoint for resistance of 1 µg/ml. The Etest (bioMerieux Inc., Durham, NC) may also be used to determine MIC, and its results correlate well with broth dilution results. While CLSI has only recommended breakpoints for clarithromycin, they have established Quality Control ranges for other agents. Because of this, Amoxicillin and Tetracycline may be tested, but the MIC value is reported without interpretation.

2.0 Clinical Significance

Helicobacter pylori is recognized as the main cause of peptic ulcer disease and a major risk factor for gastric cancer. *H. pylori* infection is also an independent risk factor for the development of atrophic gastritis, gastric ulcer disease, gastric adenocarcinomas, and gastric mucosa-associated lymphoid tissue lymphomas. Individuals infected with *H. pylori* may develop acute gastritis (abdominal pain, nausea, and vomiting) within 2 weeks following infection. The organism establishes a chronic infection in the majority of infected people, represented by chronic gastritis. Prominent mucosal inflammation is often evident in the antrum (antrum-predominant gastritis), predisposing to hyperacidity and duodenal ulcer disease.

The first-choice standard triple therapy to eradicate *H. pylori* comprises a proton pump inhibitor, clarithromycin, and either amoxicillin or metronidazole. Therapy should ideally be based on pretreatment antibiotic susceptibility testing, although this is not always practical. The main cause of failure to eradicate *H. pylori* with the standard antimicrobial regimen is clarithromycin resistance. Prevalence rates are 10 to 15% in the United States, with distinct regional variations. The clinical impact of resistance is marked, with an eradication rate for the standard therapy decreased by 70%. The key risk factor for clarithromycin resistance is previous consumption of macrolides, and prevalence of resistance after failure of treatment is extremely high.

3.0 Scope

This procedure is classified under CLIA as Highly Complex. It should be carried out by technical personnel familiarized and trained to identify *H. pylori*. Testing includes but is not limited to: morphologic recognition, confirmatory testing, and antimicrobial susceptibility testing.

4.0 Safety - Personal Protective Equipment

Performance of this procedure will expose testing personnel to biohazardous material. All specimens must be handled as potentially infectious material as outlined in the Microbiology Biohazards and Safety document.

This procedure may expose you to:

- Bloodborne and enteric pathogens

To perform this procedure, you must use:

- Gloves (specimen processing)
- Laboratory Coat
- Biological safety cabinet (specimen processing)

Disinfectant following procedure:

- Bleach dilution sprayers can be used for on demand disinfectant.

5.0 Specimen Requirements: Collection, Handling and Storage

Gastric tissue biopsy specimens are typically small in size (1-3 mm). Specimens for culture should be collected before specimens for histological examination to eliminate the risk of transferring small amounts of fixative to the container for specimens to be used for culture. *H. pylori* is a fragile organism and must be protected from desiccation and contact with atmospheric

levels of oxygen. It is important not to expose the biopsy specimens to air for any length of time and to place them either in a saline solution for short-term transport (4 h maximum) or in a bacterial transport medium for up to 1 day. Liquid Cary-Blair, normally used for transporting stool specimens for culture for enteric pathogens, may be used for transporting tissue biopsies for *H. pylori* culture. Specimens should be refrigerated during transport.

6.0 Materials

6.1 Equipment

- Incubator set at 35 ± 2°C
- Anoxomat system and jar
- PhoenixSpec Nephelometer
- Large capacity Mitsubishi AnaeroPack® System jar

6.2 Consumables

- WHIRL-PAK bag
- Laboratory marker
- Sterile transfer pipette
- Trypticase™ Soy Agar with 5% Sheep Blood (TSA II™)
- Sterile, disposable inoculating loop
- Mueller Hinton Agar with 5% Sheep Blood (use a separate plate for each Etest)
- Phoenix ID Broth
- Etest: Amoxicillin, Clarithromycin, and Tetracycline
- Sterile swabs
- BD GasPak™ EZ Campy

6.3 Control Strain

- *Helicobacter pylori* ATCC 43504

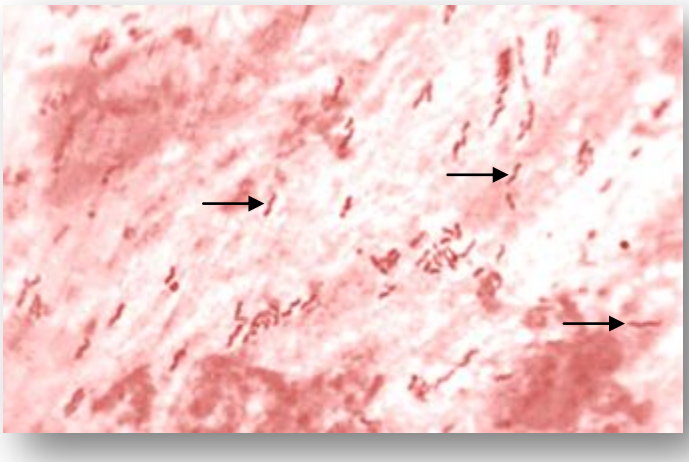
7.0 Specimen Processing

1. Retrieve a frozen stock of the *Helicobacter pylori* ATCC 43504 control strain from the -70°C freezer and allow it to thaw at room temperature while processing the gastric biopsy.
2. Perform all specimen processing in a biological safety cabinet.
3. If the biopsy was submitted in sterile saline, transfer the biopsy into a WHIRL-PAK bag along with approximately 0.5 mL of sterile saline. If the biopsy was submitted in Cary-Blair, examine the vial to locate the biopsy and transfer the biopsy to a WHIRL-PAK bag along with 0.5 mL of the Cary-Blair medium. If the biopsy is not readily visibly, pour the entire 15 mL of Cary-Blair into the WHIRL-PAK. Use a sterile transfer pipette to remove all but about 0.5 mL of the Cary-Blair back to the original vial and leave the tissue biopsy in the WHIRL-PAK.
4. Close the WHIRL-PAK by expelling excess air and folding the open end of the bag over several times.
5. Lay the WHIRL-PAK flat and gently homogenize the tissue by rolling a marker back and forth several times over the bag.
6. Once the tissue has been homogenized, open the bag and access the material with a sterile transfer pipette. Withdraw some of the homogenized material and inoculate a blood agar plate (TSA II™) with 3-4 drops. Add another drop of the material to a glass slide and heat fix on the slide warmer for Gram stain preparation.
7. Use a separate TSA II™ plate to subculture the thawed stock of the *H. pylori* control strain.
8. Aseptically streak the inoculated plates for isolation and immediately incubate under strict microaerophilic conditions in an Anoxomat jar placed at 35°C.

8.0 Direct Smear Exam

Examine the smear for gull wing-shaped, gram-negative rods.

- If curved gram-negative rods are observed report: "**Gram-negative rods resembling *Helicobacter***."



- If no curved gram-negative rods are observed report: "**No Gram-negative rods resembling *Helicobacter* seen.**"

9.0 Culture Workup and Reporting

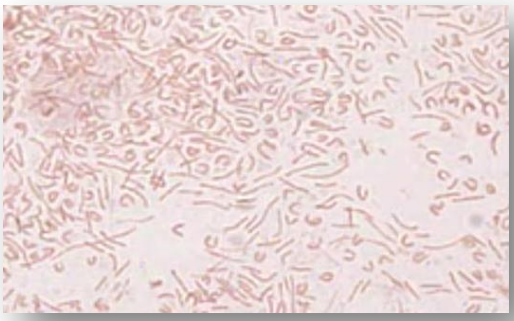
9.1 Positive Cultures

1. Incubate plates at 35 ± 2°C for 3 d before opening the jar. Some strains require as long as 7 d for growth on primary isolation. If no *H. pylori* colonies are observed on day 3, immediately return the culture plate to a microaerophilic jar and incubate until day 7.
2. *H. pylori* colonies appear small, gray, and translucent (see images below). Some strains demonstrate weak beta-hemolysis.

***Helicobacter pylori* and mixed flora**

***Helicobacter pylori* after 3 d incubation**

3. Gram stain suspicious colonies. *H. pylori* organisms from solid media appear as slightly curved gram-negative rods.



4. If curved gram-negative rods are seen in the Gram stain, perform oxidase, catalase and urea tests from colonies. *H. pylori* is positive in all three tests. The urease reaction is usually positive within several minutes.
5. Report: "***Helicobacter pylori*, Susceptibility testing in progress.**" Proceed with antimicrobial susceptibility testing as described below.

9.2 Negative Cultures

If no suspicious colonies are observed after 7 d of incubation report, "**No *Helicobacter pylori* isolated.**"

10.0 Antimicrobial Susceptibility Testing and Reporting

1. Sufficient growth is needed to prepare a suspension equivalent to a 2.0 McFarland. If necessary, prepare several subcultures on TSA II™ blood agar. The control organism should be subcultured at the same time.
2. Incubate subcultures for 3 d.
3. Retrieve the Amoxicillin, Clarithromycin, and Tetracycline Etest strips from the -20°C freezer and allow them to warm up to room temperature.
4. Using Phoenix ID Broth and a PhoenixSpec Nephelometer, prepare a suspension of the clinical isolate and the control strain. Adjust the turbidity of both suspensions to achieve a turbidity to a 2.0 McFarland standard. The suspensions should be used immediately for inoculation.
5. Dip a sterile cotton swab into the adjusted suspension of the control strain to saturate the swab. Rotate the swab several times and press firmly on the inside wall of the tube above the fluid level to remove excess fluid. Inoculate the surface of a Mueller Hinton agar plate with 5% Sheep Blood by streaking the swab over the entire agar surface. Repeat this procedure by streaking two more times, rotating the plate approximately 60° each time to ensure even distribution of inoculum. Inoculate a separate plate for each Etest. Repeat the same process for the clinical isolate suspension.
6. Apply Etest strips to the surface of the inoculated Mueller Hinton blood agar plates. Place each strip on a separate plate. Be sure that each strip is placed with the MIC scale facing upwards. Do not move the strip after it has contacted the agar surface as the antibiotic begins to diffuse immediately.
7. Place the clinical isolate and control strain susceptibility plates in a large Mitsubishi AnaeroPack® System jar along with 3 sachets of the BD GasPak™ EZ Campy (microaerophilic gas generator packs).
8. Secure the lid to the jar and incubate at 35 ± 2°C for 3 days.
9. After 3 d incubation, open the jar and examine the plates for elliptical zones of inhibition. For each antibiotic, determine where the growth intersects with the MIC scale. If the growth intersects between a two-fold dilution the MIC must be rounded up to the next two-fold value before interpretation.

10. Report the Clarithromycin MIC value with the interpretation based on the CLSI Interpretive Criteria. For Amoxicillin and Tetracycline, report the MIC value along with the comment, "**There are no CLSI Interpretive Criteria for this MIC value.**"
11. Review any ambiguous results on Rounds.

CLSI Interpretive Criteria

Antimicrobial Agent	MIC Interpretive Standards (µg/mL)		
	S	I	R
Amoxicillin	No interpretive standards		
Clarithromycin	≤ 0.25	0.5	≥ 1.0
Tetracycline	No interpretive standards		

11.0 Quality Control & Quality Assurance

- Each time a clinical sample is processed for culture, the control strain should be subcultured to a separate TSA II™ blood agar plate and included in the jar with the clinical culture. Frozen stock of *Helicobacter pylori* ATCC 43504 are available on the QC shelf in the -70°C freezer. Thaw the stock at room temperature briefly before subculture.
- When clinical isolates are recovered, two subcultures should be made to provide sufficient growth for susceptibility testing. Likewise, the control strain should subcultured to two new TSA II™ blood agar plates and incubated microaerophilically for 3 d prior to antimicrobial susceptibility testing.
- Perform susceptibility testing as described above.
- Verify that all QC results are within range before results are released for patient isolates. Refer to the table below for the CLSI QC ranges.
- Consult Rounds if QC results are out of range or difficult to interpret.
- Quality Control results must be recorded in LIS.

MIC Quality Control Ranges

Antimicrobial Agent	*Helicobacter pylori* ATCC 43504
Amoxicillin	0.015 – 0.12
Clarithromycin	0.015 – 0.12
Tetracycline	0.12 - 1

12.0 Limitations

1. *H. pylori* is a fragile organism that must be protected from desiccation and atmospheric oxygen. This applies to handling of both specimens and cultures. Improperly handled specimens or cultures will result in a failure to recover the organism.
2. Biopsy specimens for culture should be collected before samples for histological examination to prevent transferring small amounts of fixative to the culture specimen container.
3. Contaminating flora may lead to overgrowth of slower growing *H. pylori* colonies. Cultures must be carefully scrutinized on day 3. Any suspicious colonies should be subcultured for isolation before they become overgrown.

4. Placing more than one Etest on the same Mueller Hinton blood agar plate for susceptibility testing can lead to results that cannot be interpreted.

13.0 Validation Information

Helicobacter pylori ATCC 43504 and six clinical isolates were used to determine the optimal media for culture. Clinical isolates were recovered from gastric biopsies received in positive CLOTest devices. Five different agar media were evaluated including, TSA II™ Agar with 5% Sheep Blood, Campy CVA Agar, Brucella Agar with 5% Sheep Blood, Chocolate II Agar, and Modified Thayer Martin Agar. A suspension was prepared for each test isolate with a turbidity of a 0.5 McFarland standard. A separate sterile swab was dipped into each suspension and used to inoculate each of the media. Culture plates were streaked for isolation and incubated microaerophilically at 35°C for 3 days. After incubation, the jar was opened and the plates were examined for growth. All 7 (100%) of the test isolates grew best on the TSA II™ Agar with 5% Sheep Blood. The Brucella Agar also supported growth of all 7 isolates. However, the colonies were visibly smaller than those on the TSA II™ Agar. None of the test isolates grew on Campy CVA. Only one isolate grew on the Modified Thayer Martin Agar and the growth of this isolate was very poor. Likewise, the isolates grew poorly on the Chocolate Agar medium, with 3 isolates failing to produce any growth. Based on theses observations, TSA II™ with 5% Sheep Blood Agar was selected as the sole medium for clinical culture.

Helicobacter pylori ATCC 43504 and five clinical isolates were used to evaluate amoxicillin, clarithromycin, and tetracycline antimicrobial susceptibility testing by Etest. The control strain produced MIC results within the Quality Control ranges published by CLSI. Testing with the control strain was repeated four times. Results were consistently within one two-fold dilution of each other and were within the CLSI limits. MIC values were determined for the five clinical isolates. Two (40%) of the five isolates produced resistant MICs to clarithromycin. The control strain and the 5 clinical isolates were frozen and sent to Focus Diagnostics for correlation testing. Focus also performs MIC testing by antibiotic gradient diffusion. The S-I-R results reported by Focus agreed with the results obtained in house.

14.0 References

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3. CLSI. 2010. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; approved guideline – 2nd ed. Clinical Laboratory Standards Institute, Wayne, PA.
4. Mégraud F, Lehours P. *Helicobacter pylori* Detection and Antimicrobial Susceptibility Testing. Clin. Microbiol. Rev. 2007; 20(2):280-322.

15.0 Document Control History

Adopted/Reviewed by director (AR) 03/26/2013 and supervisor (JC) 04/01/2013

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