PROVIDENCE
Sacred Heart
Medical Center &
Children's Hospital

Bordetella pertussis/parapertussis Culture

Department of Microbiology

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1.0 Clinical Significance

Bordetella pertussis and *Bordetella parapertussis* are responsible for the disease, pertussis, also referred to as "whooping cough." This is a highly communicable, vaccine-preventable disease that lasts for many weeks and is typically manifested in children with paroxysmal spasms of severe coughing, whooping, and post-tussive vomiting. Major complications are most common among infants and young children and include hypoxia, apnea, pneumonia, seizures, encephalopathy, and malnutrition. Young children can die from pertussis. Most deaths occur among unvaccinated children or children too young to be vaccinated.

2.0 Purpose or Principle

Confirmation of *B. pertussis*/*parapertussis* infection may be achieved by a variety of test methods including culture, direct fluorescent-antibody, and PCR. While PCR testing is relatively rapid and offers a significantly increased sensitivity in detecting *B. pertussis/parapertussis*, culture has been used historically to aid in the diagnosis of pertussis. Culture isolation may sometimes be important for antimicrobial susceptibility testing and molecular typing during outbreaks.

Successful culture isolation of *B. pertussis/parapertussis* is dependent on many factors. Collection of good quality specimens and using specific transport devices are essential. Nasopharyngeal specimens are planted on specialized media and incubated and monitored for several days. Regan-Lowe agar is a selective medium for cultivating *B.* pertussis/*parapertussis*. It consists of charcoal agar as a basal medium supplemented with cephalexin to inhibit bacteria indigenous to the nasopharynx and defibrinated horse blood to support growth of *Bordetella*.

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 *Bordetella*. Testing includes but is not limited to: morphologic recognition, confirmatory testing, and Quality Control testing of media and reagents.

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 Providence Sacred Heart Microbiology Safety Guidelines.

The reagents and/or chemicals that are used for isolate identification may be hazardous to your health if handled incorrectly. Information concerning the safe handling of the reagents and/or chemicals used in this procedure, as well as other important safety information may be obtained by consulting the Safety Data Sheet (SDS). Before performing any part of this procedure, the technologist must take any and all precautions and adhere to all prescribed policies.

This procedure may expose you to:

- Airborne pathogens
- Hazardous reagents

To perform this procedure, you must use:

- Gloves must be worn when handling specimens.
- A laboratory coat must be worn when handling specimens, cultures, and reagents.
- A biological safety cabinet must be used when processing specimens and when making organism suspensions.

Disinfectant following procedure:

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

Reference for spill/decontamination:

- SDS
- Chemical hygiene plan

5.0 Specimen Requirements

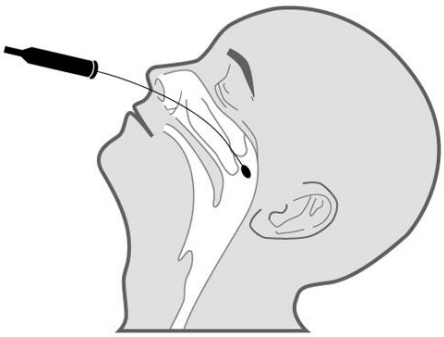
5.1 Specimen Types

Preferred specimens for laboratory diagnosis of pertussis are nasopharyngeal aspirates and posterior NP swabs. *B. pertussis* shows tropism for the ciliated epithelial cells in the nasopharynx. NP aspirates yield more positive culture results than do NP swabs.

Unacceptable specimens include swabs of the throat or nares, and lower respiratory specimens such as sputum. These types of specimens are not samples of the primary site of infection and they are often contaminated with large numbers of normal flora, which can result in overgrowth of isolation media.

5.2 Specimen Collection & Transport

5.2.1 Nasopharyngeal Swabs



When properly collected, NP specimens contain the ciliated respiratory epithelial cells. Swab specimens are collected by inserting a small swab on a flexible (plastic or aluminum wire) shaft through the nostril. The placement of the swab into the posterior nasopharynx is important. Once in place, the swab should be rotated gently for several seconds before withdrawal in order to absorb secretions. It is generally recommended that two NP swab specimens be collected, one through each nostril.

NP swabs for culture must be submitted in Amies transport gel with charcoal or, alternatively, in Regan-Lowe transport medium. Amies transport gel medium that does not contain charcoal is unacceptable for *B. pertussis* culture as it does not maintain the organism viability in the presence of nasal secretions and normal flora. NP swabs may be transported refrigerated or at room temperature but should not be frozen.

5.2.2 NP Aspirates

NP aspirates are collected by inserting a narrow catheter or infant feeding tube through the nostril to the posterior nasopharynx. A mucus trap and hand-operated vacuum pump are connected to the other end. Suction is applied while the tube is in place and while slowly withdrawing it back through the nostril. NP aspirates should be submitted in a capped sterile container. NP aspirates may be transported refrigerated or at room temperature but should not be frozen.

6.0 Materials

6.1 Equipment and/or Testing System

- Aerobic incubator (non-CO_2) set at 35 ± 2°C
- Fluorescent microscope

6.2 Consumables

- Inoculating swabs & loops
- Tape for culture plates
- Glass microscope slides

6.3 Media & Reagents

- Regan-Lowe Charcoal Agar
- Gram stain reagents
- BD Difco™ FA *Bordetella pertussis*

6.4 Control Materials

- *Bordetella pertussis* ATCC 9340
- *E. coli* ATCC 25922
- *Staphylococcus aureus* ATCC 25923

7.0 Procedure

7.1 Specimen Processing

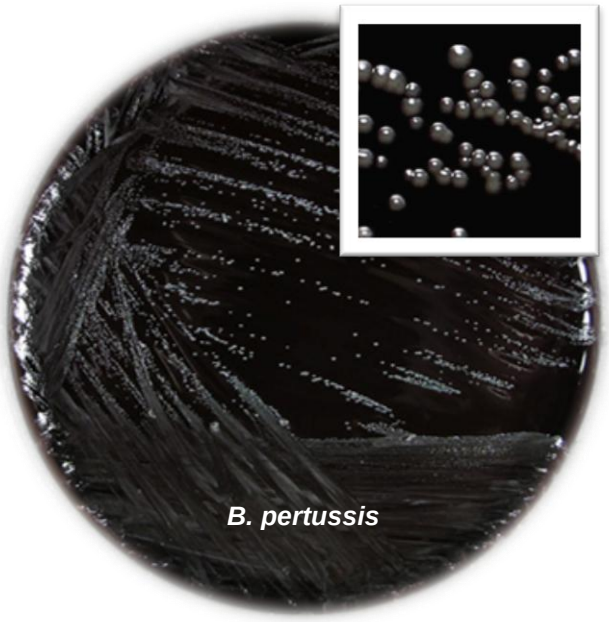
1. Upon receipt, verify that the source of the specimen submitted is NP and that it was submitted on the appropriate collection device. If a specimen is submitted from a source other than NP, or if a specimen is submitted using an incorrect transport device, the client will need to be contacted.
2. Acceptable specimens should be ordered/accessioned using the workpar, **CBPERT**.
3. Use the printed accession labels for labeling the specimen, medium for inoculation, and the Pertussis Culture Log.
4. In a biologic safety cabinet, inoculate a Regan-Lowe agar plate using the NP specimen. If a swab is received, it should be carefully rolled and pressed against the agar surface. If two swabs are received from the same patient (one from each nostril) they should both be used to inoculate the same piece of medium.
5. Using a sterile inoculating loop, streak the plate for quadrant isolation.
6. Seal the plate with tape to prevent desiccation during incubation.
7. Incubate the plate aerobically (non-CO_2) at 35 ± 2°C.

7.2 Culture Examination

The plate should be examined for suspicious colonies beginning on day 3 of incubation and daily thereafter for a total incubation period of 7 days.

7.3 Isolate Identification

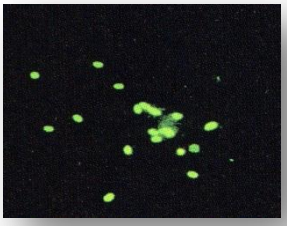
	B. pertussis	***B. parapertussis***
Colony morphology on Regan-Lowe	Colonies appear after 3-7 d and are tiny to small, round, domed, shiny, and mercury-silver colored.	Colonies appear after 2-3 d and are similar to those of *B. pertussis* but are less domed and less shiny.
Oxidase	+	–
Gram stain	Small Gram-negative rods	Small Gram-negative rods
Confirmation	DFA	Urease +, grows on BAP, and slowly on MAC, brown soluble pigment on MHA



8.0 Direct Fluorescent Antibody Stain for *B. pertussis*

Suspect colonies of *B. pertussis* should be tested with fluorescent antibody stain for confirmation. Suspension preparation and dilution should be performed in a biologic safety cabinet.

1. Using sterile saline, prepare a suspension of the test isolate equivalent to a 0.5 McFarland turbidity standard.
2. Dilute the suspension 1:100, and prepare a smear on a glass slide using the 0.01 mL calibrated loop.
3. Allow the smear to air dry. Then fix by flooding the smear for 1 min with 95% ethanol. Drain the slide, and allow to air dry.
4. Stain the smear according to the Pertussis FA Stain Procedure.



Bordetella pertussis cells should appear as small coccobacillary rods with pronounced peripheral apple green fluorescence and dark centers. Fluorescence must be intense along with the other staining characteristics and morphology

9.0 Interpretation and Reporting

9.1 Preliminary Report

If no suspicious colonies are noted on day 3, send the following report, **No *Bordetella pertussis* or *parapertussis* isolated to date.**

9.2 Final Report

Negative

- If no suspicious colonies are seen after 7 days of incubation, send the following report: **No *Bordetella pertussis* or *parapertussis* isolated.**

Positive

- If *B. pertussis* is isolated, report: ***Bordetella pertussis* isolated. This is a reportable disease. Please contact your County/State Health Department.**
- If *B. parapertussis* is isolated, report: ***Bordetella parapertussis* isolated.**

10.0 Quality Control

10.1 Media Quality Control Testing

Each new lot or shipment of Regan-Lowe should be tested using *Bordetella pertussis* ATCC 9340, *E. coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923.

1. Working in the biological safety cabinet, make suspensions of each test strain in sterile saline directly from growth on weekly subculture plates. Adjust each suspension to a turbidity equivalent to a 0.5 McFarland standard.
2. Using sterile saline, dilute the suspension 1:10.
3. Using a 0.01 mL calibrated loop (large urine loop), inoculate the agar, and streak for isolation.
4. Incubate media in ambient atmosphere at 35 ± 2°C for 72 h.
5. Enter results in LIS. If results are unacceptable, notify supervisor or technical specialist.

Expected Results for Regan-Lowe Quality Control

Control Strain	Expected Result
B. pertussis ATCC 9340	Tiny, domed, glistening, white to gray colonies
S. aureus ATCC 25923	Partial or complete inhibition
E. coli ATCC 25922	Partial or complete inhibition

10.2 Fluorescent Antibody Stain Quality Control Testing

Quality control smears should be stained each time a smear of a patient isolate is stained.

1. Control slides are prepared in batches and stored at -20°C. See Pertussis FA Stain Procedure for preparation of control smears. Allow slides to reach room temperature.
2. Fluorescent antibody stain is prepared ahead of time and stored at -20°C (see Pertussis FA Stain Procedure for details). Remove a vial of stain from the freezer, and thaw at room temperature.
3. Proceed with staining of QC slides and smear of patient isolate, following the Pertussis FA Stain Procedure.
4. Add a drop of mounting medium to the slide, and apply cover slip.
5. Examine at 100X oil immersion to determine proper staining reactions.
6. Enter results of QC testing in LIS. Notify supervisor if results are unacceptable.

Expected Results for *B. pertussis* Fluorescent Antibody Stain Quality Control

Control Strain	Expected Result
Positive Control	3-4 + fluorescence - small coccobacillary rods with pronounced peripheral apple green fluorescence and dark centers.
Negative Control	No fluorescence

10.3 Temperature Quality Control

Temperatures of all controlled instruments and environments must be checked daily and recorded in LIS. This includes refrigerators, freezers, incubators, and heating blocks. The thermometers used for measuring temperatures are checked against a NIST certified thermometer prior to being placed into use and must not vary more than $\pm$ 1° C.

11.0 Limitations

11.1 Culture

The sensitivity of culture varies greatly. Variables that affect the recovery of *B. pertussis* in culture include prior antibiotic therapy, duration of symptoms, age, and vaccination status, specimen collection and transport conditions, the type and quality of media used, and other conditions.

11.2 Fluorescent Antibody Stain

1. Some experience is required to grade the intensity of the fluorescence and to ignore nonspecific staining of gram-negative diplococci. A gram stain should be performed prior to the FA stain to ensure that the isolate is a gram-negative coccobacillus.
2. Cross reactions of variable staining intensities may occur with a small population of cells in a *B. bronchiseptica* suspension. This species of *Bordetella* typically grow within 24 h, are large, flatter, and have a dull rather than shiny appearance. *B. bronchiseptica* is also more definitively rod-shaped than *B. pertussis* on Gram stain. A positive 4-h urease test can help distinguish *B. bronchiseptica* and rule out *B. pertussis*.

12.0 Verification Information

12.1 Evaluation of Transport Media

This portion of the evaluation was performed in order to determine whether or not a separate transport device would be necessary for swab specimens submitted for *B. pertussis* culture. The standard recommendations for NP swabs are either Amies Gel with charcoal or Regan-Lowe transport media. This study compared Amies Gel, to the recommended medium, Amies Gel with charcoal. A clinical strain of *B. pertussis* and ATCC strain 9340 were used to evaluate organism viability in both transport media. Suspensions of the isolates were prepared to yield an

approximate concentration of 10^7 CFU/mL and 10^5 CFU/mL. 100 μL of each suspension was loaded onto pairs of separate swabs. This yielded swabs that were loaded with approximately 10^6 CFU and 10^4 CFU respectively. The swabs were placed into the transport media. One set of swabs was then stored at room temperature while the other set was refrigerated at 4°C. Swabs were then used to inoculate Regan-Lowe agar at specific time intervals. A separate set of swabs was used for inoculation at 0, 24, and 48 h. Plates were then incubated in ambient atmosphere at 35 ± 2°C. After 4 d of incubation, the plates were examined and compared for growth. No appreciable difference was noted between the number of colonies recovered from the time 0, 24, or 48 h swabs for either strain. No difference was noted between recoveries from Amies Gel vs. Amies Gel with charcoal. There was also no difference noted between the swabs stored at room temperature vs. those stored in the refrigerator.

The next phase of the transport media evaluation involved testing for the survival of *B. pertussis* cells in the presence of nasal secretions and competing flora. A few laboratory volunteers donated self-collected nasal swabs. These swabs were then loaded with approximately 10^6 CFU of the clinical *B. pertussis* strain and placed into transport media. The swabs were then stored at room temperature for 0, 24, and 48 h. The swabs were inoculated onto Regan-Lowe agar without cephalexin and incubated in ambient atmosphere at 35 ± 2°C for 4 d. The cultures produced varying amounts and types of normal flora. In some of the cultures, the *B. pertussis* colonies were overgrown by the competing flora. However, one of the cultures produced a few colonies of staph growing among *B. pertussis* colonies. Remarkably, there were zones of *B. pertussis* inhibition evident around the staph colonies. This staph strain was subcultured for use in additional standardized evaluation studies.

In the next phase of the transport media evaluation, the *B. pertussis* clinical strain and ATCC 9340 strain were tested along with nasal secretions and with the inhibitory staph strain. Multiple nasal swabs were collected from one donor. These were then loaded with approximately 10^6 CFU of *B. pertussis*, and the swabs were stored at room temperature for 0, 24, and 48 h. For the inhibitory staph, individual suspensions of *B. pertussis* and staph were prepared and then combined in equal amounts. The *B. pertussis* suspensions were prepared to approximate 10^7 CFU/mL and the inhibitory staph to 10^5 CFU/mL. 100 μL of the combined suspensions was then loaded onto paired swabs. Swabs were placed into both Amies Gel and Amies Gel with charcoal and stored at room temperature for 0, 24, and 48 h. The swabs were then used to inoculate selective Regan-Lowe agar and incubated in ambient atmosphere at 35 ± 2°C for 4 d. The plates were examined and compared for recovery of *B. pertussis*. The time 0 plates yielded more growth from the Amies Gel with charcoal swabs than the Amies Gel swabs. However, the colonies were too numerous to count for actual comparison. A diminishment of recovery was observed from the 24 and 48 h swabs. These plates produced countable colonies. The data from the cultures is summarized in the table below.

Colony Count of Cultures from AG vs. AG w/charcoal

Time on swab	Amies	Amies + Charcoal
Nasal Swab + 10^6 CFU of Clinical *B. pertussis* Strain		
24 h	74	321
48 h	41	109
Nasal Swab + 10^6 CFU *B. pertussis* ATCC 9340		
24 h	22	127
48 h	1	64
10^6 CFU *B. pertussis* Clinical Strain + 10^4 CFU Inhibitory Staph		
24 h	15	192
48 h	6	77
10^6 CFU *B. pertussis* ATCC 9340 + 10^4 CFU Inhibitory Staph		
24 h	21	142
48 h	2	49

Because of the significantly diminished recovery of *B. pertussis* from the Amies Gel swabs at 48 h, Amies Gel with charcoal was selected as the transport device for NP swab specimens.

12.2 Evaluation of Alternate Culture Medium

Reagan-Lowe was selected as the primary culture medium. However, RL agar is a selective medium that contains cephalexin. While this improves recovery of B. pertussis by limiting overgrowth of normal flora, some strains of B. pertussis reportedly will not grow on this medium. The recommendation is to use a non-selective medium in parallel with RL agar. RL agar without cephalexin is available. However, it is only produced by Becton Dickinson by special request and is significantly more expensive than regular RL agar. For this reason, an alternative non-selective medium was sought. There are reports of recovery of B. pertussis on BCYE agar. To evaluate this medium, a total of 21 clinical strains and 1 ATCC strain were inoculated onto both RL and BCYE agar for comparison. All 22 (100%) of the B. pertussis isolates grew equally well or better on BCYE agar than on RL. Many strains grew larger colonies more quickly on BCYE than RL.

Addendum 5/8/2012

After over 2 years of testing, no *B. pertussis* isolates were recovered on BCYE that failed to grow on RL agar. The BCYE agar was frequently overgrown with contaminating organisms and did not prove to increase the sensitivity of the test. Because of these factors, the use of BCYE for pertussis culture was discontinued.

12.3 Evaluation of Fluorescent Antibody Stain

Difco™ FA Bordetella Pertussis stain has long been in use in this lab for direct staining of NP smears. This reagent can also be used for the purpose of confirming the identity of suspect culture isolates. To evaluate the use of this reagent for confirmatory testing, a total of 11 clinical strains of *B. pertussis* were used. A suspension equivalent to a 0.5 McFarland turbidity standard was prepared for each isolate. Suspensions were diluted 1:10 to avoid dulling of the fluorescence with excess antigen. Smears were prepared from each suspension using a 0.01 mL calibrated loop. Smears were allowed to air dry and then were fixed by flooding with 95% ethanol for 1 min. Smears were stained and examined according to the Pertussis FA Stain Procedure. Smears from all 11 (100%) of the strains had brightly fluorescing coccobacilli consistent in morphology to *B. pertussis*.

13.0 References

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14.0 Document Control History

Microbiology Director Approval: Dr. Ann Robinson 03/29/2010, reviewed 12/18/2014

Microbiology Supervisor Reviews: Jerry Claridge 03/29/2010, 03/2011, 03/2013, 12/18/2014

Revisions & Updates:

05/08/2012 Discontinued nonselective medium. 12/23/2014 Added information and workup for *B. parapertussis.* Added procedure sections: Scope and Safety.