Chapter 2 — Diagnosis and Laboratory Methods

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Making a specific diagnosis of pertussis in patients with clinical evidence of infection is one of the many challenges presented by *Bordetella pertussis*. Isolation of this bacterium remains the only certain method for making a pertussis diagnosis. Efforts to carefully obtain and optimally handle specimens for culture can greatly assist in isolating *B. pertussis*. Although polymerase chain reaction (PCR) and serology have the potential to be useful in the future, the lack of standardization of these tests and their unknown correlation with pertussis illness limit their current usefulness. This section reviews the accepted laboratory methods for diagnosis of *B. pertussis* infections; antibiotic-susceptibility testing of *B. pertussis* isolates; and pulsed-field gel electrophoresis (PFGE), a genetic tool to help define the epidemiology of *B. pertussis* strains.

OBTAINING A SPECIMEN FOR DIAGNOSIS OF PERTUSSIS

All suspected cases of pertussis should have a nasopharyngeal aspirate or swab obtained for culture from the posterior nasopharynx. For *B. pertussis*, nasopharyngeal aspirates have similar or higher rates of recovery than nasopharyngeal swabs and therefore are the preferred method of specimen collection.[1] Aspirates are also better to use if another diagnostic test (e.g., PCR) is to be performed on the same specimen. Throat and anterior nasal swabs have unacceptably low rates of recovery of *B. pertussis* (see **Appendix 2-1**).[2,3] Because *B. pertussis* is fastidious and its isolation in culture is easily obscured by the growth of other nasopharyngeal organisms, optimal sampling and handling of the specimen will improve the rate of recovery (see **Chapter 1: Background**).

Nasopharyngeal aspirates are obtained by inserting a small tube (e.g., infant feeding tube) connected to a mucus trap into the nostril back to the posterior pharynx (see **Appendix 2-1**). Secretions are aspirated while the tube is in that position, and while partly withdrawing the tube. Material in the mucus trap and any material flushed from the tube are used to inoculate culture medium for isolation of *B. pertussis*.[1,2] The specimen may be split at the time of collection so that some of this material may be assayed by PCR.

Nasopharyngeal swab specimens are obtained using a Dacron™ (not cotton) swab inserted slowly through the nostril to the posterior pharynx (see **Appendix 2-1**). Ideally the swab is left in the posterior pharynx for 10 seconds before withdrawing.

CULTURE FOR ISOLATION OF *B. PERTUSSIS*

The swab or aspirate is used to inoculate culture medium for isolation of *B. pertussis*. If direct inoculation of selective medium is not possible, and although the recovery of *B. pertussis* may be compromised, clinical specimens can be placed in Regan Lowe transport medium (one half strength charcoal agar supplemented with horse blood and cephalexin). Some investigators suggest incubating the transport medium before conveying the specimen to the laboratory to provide time for growth of *B. pertussis*. This

approach may be helpful during the winter months.[4,5] The CDC Pertussis Laboratory does not recommend incubation of the specimen in the transport medium before transport to the laboratory; in our experience, incubation of the specimen in transport medium has resulted in overgrowth of other flora and decreased the yield of *B. pertussis.* Other researchers[6] and the CDC Pertussis Laboratory recommend shipping clinical specimens at 4ºC to avoid overgrowth by other bacteria.

An enriched agar medium is necessary for optimal recovery of *B. pertussis* (see **Appendix 2-2**). The CDC Pertussis Laboratory and some investigators recommend charcoal agar supplemented with 10% defibrinated horse blood and cephalexin (Regan-Lowe medium) for recovery of *B. pertussis* in clinical specimens because of its superiority over the non-selective Bordet-Gengou medium.[7] It is also advisable to routinely include a non-selective medium for primary isolation, either Regan-Lowe without cephalexin or Bordet-Gengou. Bordet-Gengou medium has the advantage of detecting the hemolysin characteristic for *B. pertussis*.

Recovery of *B. pertussis* is improved by aerobic incubation at 35ºC to 36ºC, with sufficient humidity to avoid dessication. Cultures require incubation for 3 to 4 days, and should be incubated and checked for at least 7 days; incubation up to 12 days can increase yield.[8] The methods for identifying and confirming *B. pertussis* and for distinguishing between *B. pertussis* and *B. parapertussis* are well described.[7] The two pathogens should be differentiated since *B. parapertussis* can cause a whooping cough illness that is indistinguishable from the illness caused by *B. pertussis* and pertussis vaccine may only provide limited protection against *B. parapertussis* infection.[9] Infections by other species of *Bordetella* (e.g., *B. holmesii*, *B. bronchiseptica*) are not prevented by vaccination but can be detected by culture and by some PCR methods.[9,10] Once the species of the strain is identified, patients who have infection with *Bordetella* other than *B. pertussis* do not require further public health investigation.

SUSCEPTIBILITY TESTING OF *B. PERTUSSIS* STRAINS

In recent years, a few strains of *B. pertussis* resistant to erythromycin have been recovered.[11,12] The prevalence of these strains is not known, but available evidence suggests that it is low. Cross resistance to clarithromycin and clindamycin was demonstrated in one isolate. Another isolate resistant to erythromycin was found to be susceptible to trimethaprim-sulfamethoxazole (TMP-SMX). Routine susceptibility testing is not recommended for all patients but can be considered for purposes of surveillance. Susceptibility testing to a macrolide and to TMP-SMX should be performed for strains isolated from patients who have received at least 7 days of treatment (see Chapter 3: **Treatment and Chemoprophylaxis**).

Disk-diffusion[13] (see **Appendix 2-3**) or E-Test[11] on charcoal agar containing horse blood have typically been the methods used to screen for antibiotic susceptibility of *B.*

pertussis. An air-permeable container to retain humidity is desirable; macrolide antibiotics loose potency under incubation conditions with increased concentrations of CO_2. Strains that are resistant or intermediately non-susceptible on screening should have susceptibility confirmed by agar dilution.[13] Experience determining the minimum inhibitory concentrations (MIC) by agar dilution is limited. Hoppe et. al. suggests using Mueller Hinton broth and agar with defibrinated horse blood and a high inoculum of the test bacteria.[14] Susceptibility testing is available through many state health department laboratories and at the Centers for Disease Control and Prevention (for information on shipping, see **Appendix 2-6**).

USE OF POLYMERASE CHAIN REACTION (PCR) TO DETECT *B. PERTUSSIS* AND *B. PARAPERTUSSIS* IN CLINICAL SPECIMENS

The CDC recommends culture whenever PCR is performed. Because false-positive PCR test results may occur if the swab is exposed to a *B. pertussis* DNA contaminated environment during primary inoculation of the culture medium, PCR should not be performed on the same swab used to inoculate the primary culture medium. Since two separate laboratory spaces are required (one for PCR and one for culture), a specimen should be split before it goes to the different laboratories. Specimens already obtained on only one nasopharyngeal swab may be transferred to a casamino acids solution by mixing on a vortex apparatus; the suspension can then be split in a biosafety cabinet. Swabs that have been immersed in transport medium require a DNA extraction procedure before PCR testing. Ideally, a nasopharyngeal aspirate is obtained and split at the time of collection (alternatively two nasopharyngeal swabs are obtained) and specimens are sent to the appropriate laboratories.

Numerous studies have demonstrated the potential for PCR assays to detect *Bordetella* cells with greater sensitivity and more rapidly than culture. However, no specific technique for PCR is universally accepted or validated among laboratories and the correlation between PCR results and disease is not well established. There is no quality assurance program for PCR.[15] The use of PCR without attempting culture negatively impacts monitoring for continued presence of the agent among cases of disease, recruitment of isolates for epidemiologic studies, and surveillance for antibiotic resistance. In one laboratory in western New York state, PCR was less specific than culture; outbreaks of a cough illness were falsely diagnosed as pertussis by PCR (CDC, unpublished data, 1999). The false-positive PCR results unnecessarily exposed patients to antibiotics, and used limited public health resources to control the “pertussis” outbreak. PCR is best used as a presumptive assay in conjunction with culture, under the guidelines described in **Appendix 2-4**.

TESTING NASOPHARYNGEAL SECRETIONS BY DIRECT FLUORESCEIN-CONJUGATED ANTIBODY STAINING

Although commercially available direct fluorescence antibody (DFA) tests have been

widely used to screen patients for *B. pertussis* infection, these tests lack sensitivity and specificity for *B. pertussis*.[16-18] Cross reactions with normal nasopharyngeal flora account for false-positive results in up to 85% of tests[16] and lead to substantial unnecessary public health intervention. False-negative DFA test results may delay treatment in infants (until *B. pertussis* is isolated by culture) and thereby increase morbidity.

A mouse monoclonal antibody (BL-5)-based DFA test recently became available (Accu-Mab™, Biotex Laboratories Inc, Edmonton, Canada). The technical requirements and the careful standardization necessary for previous commercially available DFA tests also apply to this new monoclonal DFA, and patients should also be cultured for isolation of *B. pertussis*. Initial evaluation demonstrated 65% sensitivity and 99% specificity when compared with culture.[19] Further testing is necessary to confirm these results. However, results from an outbreak of pertussis in Seattle during 1999 suggest the sensitivity (<30%) and specificity (~20%) of this test may be much less than initially reported (Seattle-King County Department of Public Health, unpublished data, 1999).

Health care and public health workers can weigh the benefit of a presumptive diagnosis based on one of these tests with the disadvantage of a high proportion of false-positive and false-negative results. Because of the problems with DFA tests, CDC does not recommend their use. However, if used, DFA tests require care in all technical aspects and experienced personnel for their interpretation. In addition, they should always be accompanied by culture.

SEROLOGY

Serologic tests have been used in epidemiologic investigations, and in acellular pertussis vaccine trials, but are not yet available for routine clinical use. Tests that have been used to measure serum antibodies to *B. pertussis* include enzyme-linked immunosorbent assays (ELISAs), complement fixation, agglutination, and toxin neutralization. Because ELISAs are relatively easier to perform and are less subjective, they have been used in studies to measure IgA and IgG levels to specific antigens such as filamentous hemagglutinin (FHA), pertactin (PRN), fimbrae (FIM), and pertussis toxin (PT).[20] Currently, the most generally accepted serologic criterion for diagnosis of pertussis is the use of an ELISA to demonstrate a significant increase in IgG serum antibody concentrations against PT between acute and convalescent specimens. However, because of the insidious onset of pertussis, a patient often delays seeking medical care and an acute serum specimen cannot be obtained. IgG anti-PT concentrations in convalescent sera can be a useful epidemiologic tool in investigations where the distribution of IgG anti-PT antibodies can be compared among control subjects matched to cases by age and vaccination status, and in populations with known age-related distribution of these antibodies. In Massachusetts, an IgG anti-PT ELISA has provided a useful adjunct in the diagnosis of pertussis in adolescents and adults and in documenting outbreaks of pertussis.[21] The Massachusetts State Laboratory Institute (MA SLI) identified the population-based norms for

concentrations of IgG anti-PT among persons aged ≥ 11 years.[21] A positive result was set for high specificity – the 99 percentile of the range of values from the control population; the sensitivity of a positive result was estimated to be 63%.[21] Culture should also be obtained to confirm pertussis because even some culture-positive patients may have negative IgG anti-PT ELISA results, and because *B. pertussis* isolates are useful for molecular studies. No serologic method for diagnosis of pertussis has been validated between laboratories or has been approved for diagnostic use in the U.S. The CDC and FDA are working with investigators from Vanderbilt University to validate a serologic test that could be used by other state health department laboratories in the U.S.

PULSED-FIELD GEL ELECTROPHORESIS OF *B. PERTUSSIS* STRAINS

Pulsed-field Gel Electrophoresis (PFGE) is a technique using restriction nucleases to slice the chromosomal DNA of the bacteria at specific sites, thereby creating pieces of DNA of different sizes (a type of "DNA fingerprinting"). The number of pieces and their sizes are used to identify a profile that is useful as an epidemiologic marker. Standard methods have been developed (see **Appendix 2-5**) and testing is available through State Health Department laboratories and the Centers for Disease Control and Prevention (for shipping instructions, see **Appendix 2-6**). PFGE has helped track transmission patterns within an outbreak. Epidemiologists, laboratorians, and other public health officials who are interested in using PFGE as a tool for epidemiologic studies are encouraged to have *B. pertussis* isolates saved and evaluated by PFGE.

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Appendix 2-1. Nasopharyngeal Specimen Collection

Nasopharyngeal Aspirate

1. Connect a DeeLee suction catheter of size 6 or 8 French with mucous trap to a vacuum pump or syringe with tubing that includes an in-line filter.
2. Immobilize the patient's head.
3. Gently insert the end of the catheter along the floor of the nasopharynx to the posterior pharynx. Insertion may induce coughing and tearing.
4. Apply suction by vacuum pump or syringe when the posterior pharynx is reached and maintain as the catheter is slowly withdrawn to the middle of the nasal cavity.
5. Discontinue suction and remove the catheter.
6. Flush the catheter by aspirating 0.5 - 1.0 mls of Stainer-Scholte broth or 0.1% casamino acids solution through the catheter into the trap.
7. Using a sterile nasopharyngeal swab or bacteriologic loop, apply some of the aspirated material to one quadrant of a primary culture plate.
8. Seal the ends of the trap, label the specimen and primary plate with the accession number/patient identifier. Refrigerate if not transported to the laboratory immediately.
9. Place all waste materials in a disposable biohazard bag for discard at the laboratory.

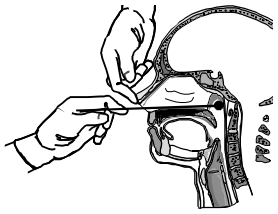
Nasopharyngeal Swab

1. Immobilize the patient's head.
2. Gently insert nasopharyngeal swab into a nostril until the posterior nares is reached.
3. Leave the swab in place for up to 10 seconds. This procedure may induce coughing and tearing. If resistance is encountered during insertion of the swab, remove it and attempt insertion on the opposite nostril.

4. Remove the swab slowly.
5. Streak one quadrant of the primary culture plate, insert the swab into the transport medium, remove the portion of the handle extending above the tube and cap the tube.
6. Label the transport tube and primary streak plates with the accession number/patient identifier and refrigerate if not transported to the laboratory immediately.
7. Place all waste materials in a disposable bag for discard at the laboratory.

Appropriate positioning of a nasopharyngeal swab

Appendix 2-2. Culture and Isolation of *B. pertussis*



1. Inoculate 2 charcoal agar plates with horse blood and cephalexin and 2 without cephalexin with the NP swab by streaking the swab across 1/3 of the plate surface. Rotate the swab while streaking. Return the swab to the transport medium.
2. Streak all plates for isolation with a bacteriologic loop.
3. Label all plates with an identifier.
4. Incubate all plates (primary and secondary) and transport tubes at 35°C with high humidity.
5. Examine plates daily for 7 to 12 days (a dissecting microscope and oblique illumination may help to visualize colonies). Small, compact, glistening colonies are characteristic of *Bordetella pertussis*. Use the quality control strain for visual comparison. Typical colonies rarely are visible before 48 - 72 hours incubation. If competing organisms threaten to overgrow the primary plate by day 2, inoculate another set of plates with the NP swab (or aspirate), which had been incubating at 35°C in the transport medium.

Appendix 2-3. Disk-Diffusion Test for *Bordetella pertussis* Sensitivity to Erythromycin (Revised from standards published by the National Committee for Clinical Laboratory Standards, 1993; Lewis, Hill).

1. Inoculate an appropriately labeled plate of charcoal agar with 10% horse blood WITHOUT CEPHALEXIN with the test culture and incubate at 35°C under

high humidity for 3 - 4 days until the first quadrant growth is confluent. Suspend cells from the charcoal agar plate in 5 ml of Mueller Hinton broth (Difco) in a screw-cap tube and adjust the turbidity to that of a 0.5 MacFarland standard.

2. Immerse a swab in the cell suspension and express excess fluid by pressing the swab tip against the side of the tube.
3. Inoculate a second labeled plate of charcoal agar with 10% horse blood WITHOUT CEPHALEXIN by streaking the swab tip over the entire plate surface in three directions while rolling the swab shaft.
4. Allow the inoculated plate to dry under ambient conditions for 10 min.
5. Aseptically apply a 15 microgram erythromycin disk to the center of the plate and gently push the disk with the tip of a sterile forceps to ensure that it adheres to the plate surface.
6. Incubate at 35°C under high humidity for 3 - 4 days until growth or zones of inhibition can be distinguished.

Inhibition zone diameters $\geq$ 42 mm indicate erythromycin sensitivity.
To date, all resistant isolates have grown to the edge of the erythromycin disk.

Appendix 2-4. Conditions for using Polymerase Chain Reaction Assays for *B. pertussis* and *B. parapertussis* as a diagnostic in a non-kit format.

1. The assay should detect and differentiate *B. pertussis* and *B. parapertussis*.

2. The laboratory performing PCR should incorporate a quality assurance program to include:
   - The physical separation of specimen processing and amplicon detection;
   - An internal control for amplification;
   - An appropriate negative control consisting of respiratory secretions (swab or aspirate, consistent with patient specimens) from a healthy individual. One negative control for every 5 - 10 clinical specimens should be processed from extraction through PCR and amplicon detection.
   - The uracil N-glycolase system to eliminate amplicon carryover;
   - A positive control consisting of the lowest DNA concentration detectable;
   - Documentation of appropriate quality assurance of each component, instrument, and reagent used in the assay;
   - Participation in a sanctioned proficiency testing program.

3. The laboratory has evaluated non-bordetellae respiratory tract normal flora and pathogens to demonstrate that specificity is 100 %;

4. The laboratory has evaluated>100 specimens with the diagnostic standard of culture to demonstrate sensitivity >70% and specificity approaching 100% with false-positive PCR results evaluated by an alternative technology or epidemiologically (the PCR false-positive rate should approach 0% for healthy individuals and patients with non-pertussis respiratory diseases);

5. Laboratory personnel performing PCR assays are blinded to patient specimens and controls;

6. Questionable results such as PCR-positive, clinically inappropriate patients (see below), or multiple consecutive positive specimens are confirmed by an alternative method;

7. For surveillance and reporting purposes, only patients who meet the CDC/CSTE pertussis clinical case definition and have a PCR-positive test can be considered to have been laboratory-confirmed (see **Chapter 11: Definitions**).

Appendix 2-5. Subtyping *Bordetella pertussis* isolates by Pulsed-Field Gel Electrophoresis of Endonuclease Restricted Chromosomal DNA (Revised 12/98 from R. Gautom, et al, 1997, J. Clin Microbiol, *35*:2977-2980)

Preparation of Gel Plugs

1. Harvest *B. pertussis* cells after 3-5 days growth on charcoal horse blood agar (WITHOUT CEPHALEXIN) into 2.0 ml of Cell Suspension TE Buffer.*
2. Adjust the turbidity of the cell suspension (0.48-0.54 for Dade Microscan Turbidity Meter; 20% transmission on a bioMerieux Vitek colorimeter; OD_{640}=± 1.40 on a spectrophotometer).
3. Transfer 200 µl of each cell suspension to 1.5 ml microcentrifuge tubes and perform the following procedures for each individual tube before proceeding:
   - Add 10 µl of proteinase K solution* (thaw, maintain on ice until use) and mix by gentle inversion 6 times.
   - Immediately add 200 µl plug agar solution* at 55°C and mix without aerosolizing by aspirating and expelling 200 µl volumes with pipet and tip.
   - Immediately dispense cell/plug agar suspension into 2 wells of a disposable plug mold (BioRad).
4. Incubate plugs at ambient temperature until solid, about 15 min.
5. Combine 1.5 ml of EDTA/Sarcosine (ES Buffer)* and 40 µl of proteinase K solution (thaw, maintain on ice until use) in a 2 ml round-bottom microcentrifuge tube (round bottom helps prevent plugs breaking).
6. Transfer the 2 plugs from each strain to the solution in the 2 ml round bottom microcentrifuge tube.

7. Immerse plug tubes horizontally in a shaking (about 75 strokes per min for reciprocating shaker) water bath at 55°C and incubate for 1 h.

Washing Plugs

1. Prepare a disposable 50 ml conical centrifuge tube with the green screen-cap (BioRad) for each pair of plugs.
2. Heat 15 ml sterile distilled water and 35 ml Tris/EDTA (Plug Wash Buffer) * per plug pair to 50°C
3. Decant the ESP Buffer from the microcentrifuge tube and slide the two plugs onto the green screen-cap.
4. Use a spatula to slip the two plugs into the centrifuge tube.
5. Immediately add 10 ml of 50°C sterile distilled water to the plugs in the centrifuge tube, (plugs must be suspended in the water and not adhering to the side of the tube).
6. Incubate in reciprocating water bath (about 150 strokes per min) at 50°C for 15 min.
7. Wash plugs in centrifuge tube five times.
   - Remove and discard the distilled water or Plug Wash Buffer from the centrifuge tube and add 10 ml of 50°C Plug Wash Buffer.
8. Incubate in reciprocating water bath (about 150 strokes per min) at 50°C for 20 min.
   - Remove and discard the final wash volume of Plug Wash Buffer from the centrifuge tube.
9. To maintain up to 4 months before restriction digestion, transfer plugs to 2 ml cryovials and add 1.5 ml of Plug Wash Buffer, and store at 4°C .

Endonuclease Restriction

1. Sterilize a razor blade and a clean glass slide with 70% isopropanol.
2. Remove a plug from the Plug Wash Buffer and place it on a clean and sterile glass slide.
3. Cut two 1 mm wide sections from the short side of the plug with the sterile razor (thinner sections yield sharper fragment bands on PFGE).
4. Transfer the two sections to a labeled 1.5 ml microcentrifuge tube and replace the remainder of the plug in 1.5 ml of Plug Wash Buffer and store at 4°C.
5. Add 100 µl of *Xba* µl I Restriction Solution Buffer* and mix gently by aspirating and expelling 100 µl volumes with pipet and tip.
6. Incubate plug suspension at 37°C for 1.5 h.

Pulsed-Field Gel Electrophoresis

1. Assemble gel box and cooling system.
2. Cool 2 L 0.5XTris/Borate/EDTA (10X TBE diluted 1:20) Buffer* to 14°C in gel box.

3. Prepare buffered 1% SeaKem Gold Agarose* and place molten agarose in 50 °C water bath until use.
4. Place plug sections and MW standard on comb (15 well, 14 cm, 1.5 mm) teeth in desired order and incubate for 20 min at ambient temperature (unused sections can be maintained in Plug Wash buffer at 4°C).
5. Place comb with plug sections in 14 X 13 mm casting stand and add 98 ml agarose, retaining 2 ml to fill wells after removal of comb.
6. Allow agarose to solidify about 20 min at ambient temperature.
7. Remove the comb.
8. Seal wells with remaining agarose.
9. Set the following conditions and start electrophoresis:
   - Initial Switch Time..... 2.16 s
   - Final Switch Time..... 35.07 s
   - Run Time..... 18.00 h
   - Angle..... 120°
   - Gradient..... 6.0 V/cm
   - Temperature..... 14°C
   - Ramping Factor..... linear
10. Remove gel from box and stain.
    - Incubate gel in 250 ml of ethidium bromide stain solution* at ambient temperature for 25 min on a rocking apparatus.
    - Wash 3 times.
      -- Remove Ethidium Bromide Solution and discard safely.
      -- Add 250 ml distilled water and incubate at ambient temperature for 30 min on a rocking apparatus.
11. Examine and photograph gel under UV illumination with appropriate safety equipment.

Reagents

- N-Lauroyl-Sarcosine ($C_{15}H_{28}NO_3Na$) *Sigma*
- Sodium Hydroxide (NaOH) pellets, *Aldrich*
- 1M Tris-HCL, pH 7.5, *Gibco*
- Trizma Base ($C_4H_{11}NO_3$), *Sigma*
- Boric Acid (H_3BO_3) *Sigma*
- EDTA ($C_{10}H_{14}N_2O_8Na_2$ $2H_2O$) *Sigma*
- SDS ($CH_3(CH_2)_{11}OSO_3Na$) *Aldrich*
- Proteinase K, 20 MG/ML Solution, *Amresco*
- Gold Agarose for PFGE, *SeaKem*
- InCert Agarose, *FMC BioProducts*
- Ethidium Bromide Solution 10 mg/ml , *BioRad*
- Restriction Enzyme *Xba I, Boehringer Mannheim*
- SuRE/Cut Buffer H for Restriction Enzymes, *Boehringer Mannheim*

- PFGE Marker 1 Lambda Ladder, *Boehringer Mannheim*

Stock Solutions

1. 0.5 M EDTA pH 8.0
   - Weigh 186.1 grams of $Na_2EDTA\text{-}2\ H_2O$
   - Dissolve in 800 mls of distilled water
   - Adjust pH to 8.0 with 10 N NaOH
   - Dilute to 1000 mls with distilled water
   - Autoclave for 15 minutes, store at room temperature
2. 20% SDS
   - Weigh 20 grams of SDS
   - Dilute in 100 mls of distilled water
   - Store at room temperature
3. 10X TBE (Tris Borate EDTA)
   - (0.9M Tris base, 0.9M Boric acid, 0.02M EDTA pH 8.0)
   - Weigh 108 grams Trizma base
   - Weigh 55 grams Boric acid
   - Add 40 mls of 0.5M EDTA, pH 8.0
   - Dilute to 1L with distilled water
   - Filter sterilize, store at room temperature
   - Discard if precipitate forms
4. 10 N NaOH
   - Weigh 200 grams of NaOH
   - Dissolve in 400 mls of distilled water
   - Cool solution to room temperature
   - Dilute to 500 mls with distilled water
   - Store at room temperature

Working Solutions

1. Cell Suspension TE (Tris-EDTA) Buffer
   - (100 mM Tris pH 8.0 and 100 mM EDTA, pH 8)
   - 10 ml of 1M Tris, pH 8.0
   - 20 ml of 0.5 EDTA, pH 8.0
   - Dilute to 100 ml with sterile distilled water
   - Filter sterilize, store at room temperature
2. 1.6% InCert/SDS Agarose Mix (1 ug PLUG AGAR)
   - (1.6% InCert, 1% SDS)
   - Weigh 0.16 grams of InCert agarose
   - Add 10 ml of sterile distilled water
   - Melt agarose in microwave (avoid boil over)
   - Add 500 µl of 20% SDS and mix well by inverting by hand. For initial use, reheat if necessary, for subsequent uses, melt in microwave and cool to 55°C in water bath.
   - Store at room temperature

3. ES Buffer
   - (0.5 M EDTA, pH 9.0; 1% sodium-lauroyl-sacrosine)
   - Weigh 93.05 grams of EDTA
   - Add to 350 ml of warmed distilled water in beaker with stir bar
   - While stirring, add NaOH pellets until pH is 8.5, adjust to 9.0 with 10 N NaOH
   - Cool to ambient temperature and re-check and adjust pH if necessary
   - Add 5 grams of sodium-lauroyl-sarcosine
   - Adjust volume to 500 mls
   - Filter sterilize, store at room temperature
4. Plug Wash Buffer
   - (10.0 mM Tris, and 1 mM EDTA, pH 8.0)
   - 10 ml 1M Tris, pH 8.0
   - 2 ml 0.5M EDTA, pH 8
   - Dilute to 1 L sterile distilled water
   - Filter sterilize and store at ambient temperature
5. 1% SeaKem Gold Agarose
   - Weigh 1 gram of SeaKem Gold Agarose
   - Dissolve in 100 mls of 0.5X TBE
   - Heat in microwave until crystals fully dissolved, avoid boil over.
   - Cool to 50˚C in water bath
6. *Xba*I Restriction Solution Buffer
   - 3 µl (30U) *Xba*I
   - 87 µl Distilled water
   - 10 µl of 10X manufacturer's recommended buffer

Appendix 2-6. Storage and Shipping *Bordetella pertussis* Isolates to the CDC Pertussis Laboratory

Storage:

1. If culture is more than 5 days old, subculture. Prepare two plates if the isolate is to be stored both at CDC and on site.
2. Use the growth from one plate to prepare each vial of storage beads (Microbank™, Pro-Lab Diagnostics, Canada) according the manufacturer's directions.
3. When confluent growth is apparent in the first quadrant, label the vials of storage beads and broth with the laboratory accession number/patient identifier.
4. Use a bacteriological loop to aseptically harvest ALL growth from a plate of medium.
5. Aseptically remove the cap from the transport agar tube and inoculate the bead suspension broth.
6. Discard the loop, tightly replace the vial cap and invert the vial 5 times (DO NOT MIX BY VORTEX APPARATUS).

7. Aspirate the suspending fluid and aseptically discard it.
8. Store the inoculated vial at -70°C.

Shipping

1. Pack the inoculated transport agar tube(s) or cryovials in the protective transport tube.
2. Pack ice packs around the protective tube in a IATA-approved shipping container.
3. Place the completed identifying and lab request forms in the shipping container.
4. Address container to:

   Attn: Epidemic Investigations Laboratory
   Meningitis and Special Pathogens Branch
   Centers for Disease Control and Prevention
   5-240 (D-11) 1600 Clifton Road
   Atlanta, GA 30333
   Phone number: 404-639-3024 or 404-639-1231
5. Maintain record of culture shipped and shipping information.
6. Prior to shipping, please call the CDC Pertussis Laboratory (Epidemic Investigations Laboratory) so that they will be expecting specimens.