Standard Operating Procedure for Real-time PCR Detection and Identification of Bordetella pertussis, B. parapertussis, and B. holmesii using AB7500

Adria D. Lee,Pamela K. Cassiday,Lucia C. Pawloski,Kathleen M. Tatti,Monte D. Martin,Elizabeth C. Briere,M. Lucia Tondella,Stacey W. Martin,The Clinical Validation Study Group

Abstract

The appropriate use of clinically accurate diagnostic tests is essential for the detection of pertussis, a poorly controlled vaccine-preventable disease. The purpose of this study was to estimate the sensitivity and specificity of different diagnostic criteria including culture, multi-target polymerase chain reaction (PCR), anti-pertussis toxin IgG (IgG-PT) serology, and the use of a clinical case definition. An additional objective was to describe the optimal timing of specimen collection for the various tests.

Clinical specimens were collected from patients with cough illness at seven locations across the United States between 2007 and 2011. Nasopharyngeal and blood specimens were collected from each patient during the enrollment visit. Patients who had been coughing for ≤ 2 weeks were asked to return in 2-4 weeks for collection of a second, convalescent blood specimen. Sensitivity and specificity of each diagnostic test were estimated using three methods—pertussis culture as the “gold standard,” composite reference standard analysis (CRS), and latent class analysis (LCA).

Overall, 868 patients were enrolled and 13.6% were *B. pertussis* positive by at least one diagnostic test. In a sample of 545 participants with non-missing data on all four diagnostic criteria, culture was 64.0% sensitive, PCR was 90.6% sensitive, and both were 100% specific by LCA. CRS and LCA methods increased the sensitivity estimates for convalescent serology and the clinical case definition over the culture-based estimates. Culture and PCR were most sensitive when performed during the first two weeks of cough; serology was optimally sensitive after the second week of cough.

Timing of specimen collection in relation to onset of illness should be considered when ordering diagnostic tests for pertussis. Consideration should be given to including IgG-PT serology as a confirmatory test in the Council of State and Territorial Epidemiologists (CSTE) case definition for pertussis.

Citation: Adria D. Lee,Pamela K. Cassiday,Lucia C. Pawloski,Kathleen M. Tatti,Monte D. Martin,Elizabeth C. Briere,M. Lucia Tondella,Stacey W. Martin,The Clinical Validation Study Group Standard Operating Procedure for Real-time PCR Detection and Identification of Bordetella pertussis, B. parapertussis, and B. holmesii using AB7500. **protocols.io**
dx.doi.org/10.17504/protocols.io.kvxcw7n
Published: 20 Nov 2017

Guidelines

Title: Standard Operating Procedure for Real-time PCR Detection and Identification of *Bordetella pertussis*, *B. parapertussis*, and *B. holmesii* using AB7500.

Purpose: To describe the procedures used for the qualitative detection of *Bordetella pertussis*, *B. parapertussis*, and *B. holmesii* DNA extracted from clinical specimens or culture isolates.

Before start

- **Disclaimer:** Names of vendors or manufacturers are provided as examples of suitable product sources; inclusion does not imply endorsement by the Centers for Disease Control and Prevention or the Department of Health and Human Services.

Reagents: (1) IS*481* primer/probe set; (2) pIS*1001* primer/probe set; (3) hIS*1001* primer/probe set; (4*)* *ptxS1* primer/probe set; (5) *RNaseP* primer/probe set; (6) PerfeCTa® qPCR ToughMix, UNG for IS*481*, *ptxS1* and *RNaseP*; Quanta Biosciences catalog number 95138-250 for 250 reactions (Storage 2-8°C for up to 6 months or 20°C for two years); (7) PCR grade water; (8) *B. pertussis* positive control DNA (for *ptxS1* assay; CDC isolate A639; for IS*481*/pIS*1001*/hIS*1001* assay ACF which consists of a mixture of A639 *B. pertussis*, F585 for *B. parapertussis* and C690 for *B. holmesii*); and, (9) Human genomic positive control DNA (for *RNaseP* assay; Applied Biosystems; catalog number 4312660; 10ng/µl).

Equipment/Materials: Microcentrifuge; Pipettes; AB7500 regular and software (Applied Biosystems); Eppendorf centrifuge 5810 or similar instrument to centrifuge; Plates 96-well (Applied Biosystems; catalog #N801-0560); Optical caps (Applied Biosystems; catalog #4323032); Aerosol barrier pipette tips; Sterile 1.5 mL microcentrifuge tubes to prepare master mixes; Pressure sensitive film-not optical film-Falcon; catalog number 353073; Aluminum foil; Bleach; Ethanol; DNA Away

Quality Control: (1) When testing clinical specimen DNA, include the *RNaseP* set to serve as an internal positive control for the assay. A poor *RNaseP* signal may indicate inhibition, low DNA yield from the DNA extraction process, or poor specimen collection. (2) Extracts from sterile swabs or sterile water (blanks) should be tested to ensure there was no cross-contamination during the DNA extraction. (3) A non-template control (NTC) using sterile water in the place of DNA should be included in each assay to ensure there was no cross-contamination during PCR set-up. This NTC also serves as a negative control. (4) DNA extracted from *B. pertussis* strain CDC A639 at low concentration should be included as a positive control for the *ptxS1* assay. DNA extracted from *B. pertussis* strain CDC A639, from *B. parapertussis* strain F585 and from *B. holmesii* strain C690 (ACF)

are combined based on the genomic equivalent of the particular species and are included as a positive control at low concentrations for IS*481*/pIS*1001*/hIS*1001* assay. The combined positive control is called ACF.

Out-of-control results: For all three assays: (1) Positive reaction of blanks indicates that contamination occurred during the DNA extraction process and specimens must be re-extracted for confirmation. Any positive specimens that were extracted at the same time as a positive blank should be regarded with suspicion. Additional epidemiological evidence is required before regarding these specimens as true positives. (2) Positive reaction of NTC – This indicates that there was contamination during the PCR set-up. The assay must be repeated. For the IS*481*/pIS*1001*/hIS*1001* and *ptxS1* assays: (3) Negative reaction of positive control indicates either that the PCR did not work or that the positive control is no longer usable. Repeat the assay using a fresh positive control extract before reporting results. If there is still a problem, test extracts again with a new prepared primer/probe set. For the *RNaseP* assay: (4) Negative or late cycle positive *RNaseP* in undiluted DNA extracts indicates that either the specimen extraction contains inhibitors to the PCR assay or that the specimen was not collected correctly. To determine if inhibitors are present, check the DNA extracts at 1:5 dilution. If the *RNaseP* results improve with the diluted extracts, the IS*481*/pIS*1001*/hIS*1001* and *ptxS1* assays must be repeated using the diluted extracts. If the *RNaseP* results do not improve, this may indicate that the NP aspirate or swab was not collected or stored as recommended.

Protocol

Step 1.
Aliquot undiluted primers and probe and store for up to 6 months at -20ºC. Cover probe aliquot tubes with foil or use dark colored tubes.

Step 2.

Prepare IS*481* primers and probe: Prepare 30 µM (10X solution) concentrations of primers and 90 µM (10X solution) probe using sterile PCR grade water. Dispense 10X working concentrations into 10-reaction aliquots and store at -20ºC. Prepare 3 µM working concentrations of primers and 9 µM working concentration probe by diluting the 10X solution to 1:10 using sterile PCR grade water just before running the assay.

Step 3.

Prepare pIS*1001* primers and probe: Prepare 90 µM (10X solution) concentrations of primers and 30 µM (10X solution) concentrations probe using sterile PCR grade water. Dispense 10X working concentrationsinto 10-reaction aliquots and store at -20ºC. Prepare 9 µM working concentrations of primers and 3 µM working concentration of probe by diluting the 10x solution to 1:10 using sterile PCR grade water just before running the assay.

Step 4.

Prepare hIS*1001* primers and probe: Prepare 30 µM (10X solution) concentrations of primers and probe using sterile PCR grade water. Dispense 10X working concentrations into 10-reaction aliquots

and store at -20ºC. Prepare 3 µM working concentrations of primers and probe by diluting the 10X solution to 1:10 using sterile PCR grade water just before running the assay.

Step 5.
Thaw working concentrations of primers and probe and mix gently with a pipette.

Step 6.

Quick spin for 5 sec.

Step 7.
Add 0.84 µl per reaction of each working concentration of each primer and probe to 1.5 ml tube (PCR mix tube).

Step 8.
Add 1 µl of PCR-grade water per reaction to the PCR mix tube.

Step 9.
Mix PerfeCTa ® qPCR ToughMix, UNG by pipetting up and down, then add 12.5 µl of the 2X master mix per reaction to the PCR mix tube.

Step 10.
Mix by pipetting and quick spin.

Step 11.
Dispense 23 µl of master mix to each test well.

Step 12.
Add 4 µl of water and 21 µl of master mix to NTC well.

Step 13.
Add 4 µl of sample DNA to the appropriate wells and close with optical caps. All specimens should betested in duplicate. A third reaction should be performed with 1:5 dilution of the DNA extract.

Step 14.
Add 4 µl of positive control dilutions and seal with the optical cap.

Step 15.
Briefly centrifuge 96-well plate and confirm that reaction mixes are at the bottom of the wells, with no bubbles present.

Step 16.
Place the 96-well plate in the AB7500 and close tray. Start run.

Step 17.

ptxS1 Primer and Probe Reagent preparation: Prepare 70 µM (10X solution) concentrations of primers and 30 µM (10X solution) concentrations of probe using sterile PCR grade water. Dispense 10X working concentrations into 10-reaction aliquots and store at -20ºC. Cover probe aliquot tubes with foil or use dark colored tubes. Do not store aliquots in frost-free freezers and do not re-freeze thawed aliquots. Prepare 7 µM working concentrations of primers and 3 µM working concentration of probe by diluting the 10X solution to 1:10 using sterile PCR grade water just before running the assay.

Step 18.
Thaw working concentrations of primers and probe and mix gently with a pipette.

Step 19.

Quick spin for 5 sec.

Step 20.
Add 2.5 µl per reaction of each working concentration of each primer and probe to a 1.5 ml tube (PCR mix tube).

Step 21.
Add 1 µl of PCR-grade water per reaction to the PCR mix tube.

Step 22.
Add 1 µl of PCR-grade water per reaction.

Step 23.
Mix PerfeCTa ® qPCR ToughMix, UNG by pipetting up and down, then add 12.5 µl of the 2X master mix per reaction to the PCR mix tube.

Step 24.
Mix by pipetting and quick spin.

Step 25.
Dispense 21 µl of master mix to each well.

Step 26.
Add 4 µl of water to NTC well.

Step 27.
Add 4 µl of sample DNA to the appropriate well and close well with an optical cap. All specimens should be tested in duplicate. A third reaction should be performed with 1:5 dilution of the DNA extract.

Step 28.
Add 4 µl of positive control to final well and reseal with the optical cap.

Step 29.
Briefly centrifuge 96-well plate and confirm that reaction mixes are at the bottom of the wells, with no bubbles present.

Step 30.
Place the 96-well plate in the AB7500 and close tray. Start run.

Step 31.

RNaseP Primer and Probe Reagent Preparation: Prepare 4 µM working concentrations of primers and 1 µM working concentration of probe using sterile PCR grade water. Dispense working concentrations into 10-reaction aliquots and store in dark for up to 6 months at -20ºC.

Step 32.
Thaw working concentrations of primers and probe and mix gently with a pipette.

Step 33.

Quick spin for 5 sec.

Step 34.
Add 2.5 µl per reaction of each working concentration of each primer and probe to a 1.5 ml tube (PCR mix tube).

Step 35.
Add 1 µl of PCR-grade water per reaction.

Step 36.
Mix PerfeCTa ® qPCR ToughMix, UNG by pipetting up and down, then add 12.5 µl of the 2X master mix per reaction to the PCR mix tube.

Step 37.
Mix by pipetting and quick spin.

Step 38.
Dispense 21 ul of master mix to each well.

Step 39.

Add 4 µl of water to NTC well.

Step 40.
Add 4 µl of sample DNA to the appropriate well and close wells with an optical cap.

Step 41.
Add 4 µl of positive control to final well and close with an optical cap.

Step 42.
Briefly centrifuge 96-well plate and confirm that reaction mixes are at the bottom of the wells, with no bubbles present.

Step 43.
Place the 96-well plate in the AB7500 and close tray. Start run.

Step 44.

Interpretation: The NTC reactions should not exhibit background. A cycle threshold (Ct) value in the NTC control indicates sample contamination during PCR set up. The *B. pertussis* DNA positive control (A639) should be positive for both IS*481* and *ptxS1*and negative for pIS*1001* and hIS*1001*. A negative result for either IS*481* or *ptxS1* means that either the control DNA is old or the PCR was not set up correctly. A positive result for either pIS*1001* or hIS*1001* means that contamination of the control occurred or the PCR was not set up correctly. In either case, the assay must be repeated. The human genomic DNA positive control should be positive for *RNaseP*. A negative result means that either the control DNA is old or the PCR was not set up correctly. The blank swabs or sterile water should be negative. If they are not, this indicates that there was contamination during the DNA extraction step and all positive specimens must be re-extracted. All clinical specimens (not isolates) should be positive for *RNaseP*. When the above controls are met, any specimen that is positive for two of the three replicates (two reactions with undiluted DNA extract and one reaction with DNA diluted 1:5) for the IS*481* assay and that is positive for one of the three replicates (two reactions with undiluted DNA extract and one reaction with DNA diluted 1:5) for *ptxS1* assay is considered positive for the presence of *B. pertussis*. If a specimen is negative in the IS*481* assay, positive in both the *ptxS1* assay and the pIS*1001* assay the combined result is considered positive for *B. parapertussis*. If a specimen is positive in the IS*481* assay in at least two of the three replicates, positive for hIS*1001* and negative in the *ptxS1* assay it is positive for *B. holmesii*. Note: Care should be taken when using this algorithm to interpret high Ct values (35 cycles or greater) IS*481* positive / *ptxS1* negative clinical specimens. Since IS*481* is present in many copies and *ptxS1* is only present in one copy, a clinical specimen that is late cycle IS*481* positive (Ct values 35 or greater) and *ptxS1* negative may indicate the presence of low levels of *B. pertussis*. Note: In outbreak settings, in particular, when many specimens are handled in a short period of time, low levels of *B. pertussis* DNA detected by high cycle IS*481* positive and *ptxS1* negativemay be due to DNA contamination during specimen collection, DNA extraction, or PCR set up. In this case the PCR result should be interpreted as indeterminate if not confirmed by another

laboratory test such as culture or serology. Epidemiological and clinical relevance of high IS*481* positive PCR tests should also be taken into consideration. It is also important to keep a record of the Ct values of all reactions in a database in order to facilitate results interpretation.

Step 45.

Limitations: (1) If inhibitors are present in a DNA extraction, PCR assays may produce a false negative result. If inhibition of the *RNaseP* assay is noted for a clinical specimen, extracted DNA should be tested at 2 or more dilutions (e.g., 1:5 and 1:10) to verify the result. (2) Data suggest that clinical specimens collected subsequent to initiation of antimicrobial treatment may not be positive for *Bordetella* spp. due to reduction of organisms. Whenever possible, specimens collected prior toadministration of antimicrobial agents should be used to determine infection with *Bordetella* spp. (3) *RnaseP* should not be performed if specimen was transported in Regan Lowe transport medium containing horse blood, as cross-reactivity has been observed.