Pitfalls in the Validation of Molecular Assays for *Bordetella pertussis*

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Objectives

- List *Bordetella* genetic targets that have been utilized in PCR-based diagnostic tests
- Compare advantages and disadvantages of multi- and single-copy *Bordetella* targets
- Discuss causes of false-positive *B. pertussis* tests
- Describe relevance of *Bordetella* species other than *B. pertussis*

Disclosures

- Will not be discussing any specific RUO tests, or off-label use of IVDs
- Have received grants from BioFire Diagnostics

Microbiology—*Bordetella* spp.

- 1994: 4 species
  - *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, *B. avium*
- By 1999, 3 additional species
  - *B. hinzii*, *B. holmesii*, *B. trematum*
- 2007
  - *B. petrii*
- Gram-negative, aerobic* bacillus. Varying growth requirements
  *B. petrii* can grow under anaerobic conditions

Clinical Significance of *Bordetella* spp.

- Respiratory
  - *B. pertussis*
  - *B. parapertussis*
  - *B. bronchiseptica*
  - *B. holmesii*
- Bacteremia
  - *B. bronchiseptica*
  - *B. hinzii*
  - *B. holmesii*
- Wounds, otitis
  - *B. trematum*

Epidemiology of Pertussis

- 27,000+ cases reported in U.S. in 2010 (CDC)
- Estimated 800,000+ cases in U.S., and 50 million worldwide, annually (CDC and WHO)
- Estimated 300,000 deaths worldwide, annually (WHO)
Epidemiology of Pertussis

Reservoir- untreated, symptomatic persons (esp. adolescents, adults). No animal reservoir
Spread by respiratory droplets (close contact required)
Highly contagious (80-90% of susceptible contacts infected)
No carrier state, but asymptomatic infections
Endemic, with epidemics every 3-5 years

Re-emergence of Pertussis

- Increased use of NAATs; more sensitive than traditional methods
- Recognition of disease in adolescents and adults
  - Serosurveys in 1980’s and 90’s indicated that up to 25% of prolonged cough illness in adolescents and adults due to B. pertussis
  - Immunity from prior infection or vaccination is not lifelong
  - Usually atypical presentation
- Increased reporting

Reported Pertussis in U.S.—1940-2002

Pertussis

- Classical
  - Catarrhal stage (1-2 wk): typical URI, fever
  - Paroxysmal stage (1-6 wk): paroxysmal cough, whoop, posttussive vomiting
  - Convalescent stage (2 wk-mo): gradual resolution of cough
- Atypical
  - Severe cough; several wk’s duration; whoop, posttussive vomiting absent
  - Differential includes C. pneumoniae, M. pneumoniae, viruses
Laboratory Diagnosis

• Direct fluorescent antibody staining
• Culture
• Serology
• Nucleic acid amplification

Trends in Laboratory Tests for B. pertussis


Source: CDC

Nucleic Acid Amplification

• More sensitive than culture
  — Sensitivity affected by duration of symptoms and patient age
  — After a couple weeks of cough, serology is more sensitive
• Persons exposed but not symptomatic can be positive
• Non-PCR molecular methods
  — Loop-mediated isothermal amplification
• One FDA-cleared test (FilmArray Respiratory Panel; BioFire Diagnostics)

Affect of Patient Age and Duration of Disease on PCR Positivity

Source: van der Zee et al. 1996. J Infect Dis. 174:89-96

Validation and Verification

• Definitions vary; involve the regulatory requirement for up front demonstration of test’s performance characteristics
  — FDA-cleared tests, unmodified
  — FDA-cleared, modified
  — Laboratory-developed tests
• CAP Microbiology checklist (molecular section)
  — Verification of unmodified FDA-cleared tests
  — Validation of laboratory-developed or modified tests*

*Bordetella pertussis LDTs

• More extensive validation process (as per CLIA, must establish performance characteristics)
  — In addition to diagnostic accuracy, reproducibility, reportable and reference ranges:
    • Analytical specificity, incl. interfering substances
    • Analytical sensitivity (limit of detection)
Validation of Molecular Assays for *B. pertussis*

- Target selection
  - *B. pertussis* specific
  - Detection of *B. parapertussis*
  - What about *B. holmesii*?
  - Multi-copy versus single copy
- Analytical specificity
  - *B. holmesii*
  - *B. bronchiseptica*
- Analytical sensitivity versus diagnostic sensitivity
  - Can a test be too sensitive?

Validation Pitfalls—Analytical Specificity

- *B. bronchiseptica*
  - Possesses various insertion sequences
  - Often present in rare isolates.
  - Varies by human vs. animal source
  - Need large number and diversity of isolates to rule out cross-reactivity
  - How important is it anyway? (Rarely isolated from humans)

Validation Pitfalls—Analytical Specificity

- *B. parapertussis*
  - 2–20% of *Bordetella* isolates; esp. infants
  - More likely to present as nonspecific cough

What About *B. holmesii*?

- Isolated cases, or outbreak associated?
- Outbreak of pertussis illness in Ohio 2010-2011
  - Specific *Bordetella* species identified in 164 specimens
    - 68% positive for *B. pertussis*
    - 29% positive for *B. holmesii*
    - 2% positive for both species

P2P Transmission of *B. holmesii*?

- Co-transmission with *B. pertussis*; Columbus, OH 2010-2011
- Evidence of P2P transmission in Japan, 2010-11
  - Junior high school students (again, adolescents) and teachers
  - *B. holmesii*-specific recA PCR

Source: Kamiya et al. 2012 Emerg Infect Dis. 18:1166-9
**B. pertussis** Gene Targets

- Insertion sequences
- Single-copy targets

**Insertion Sequences**

<table>
<thead>
<tr>
<th>Insertion sequence</th>
<th>B. pertussis</th>
<th>B. parapertussis</th>
<th>B. bronchiseptica</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS6110</td>
<td>+ / &gt;50</td>
<td>- / NA</td>
<td>- / 8.10</td>
<td>(11, 28, 31)</td>
</tr>
<tr>
<td>IS1001</td>
<td>- / NA</td>
<td>+ / 20</td>
<td>- / NA</td>
<td>(17 / 1 / 7)</td>
</tr>
<tr>
<td>IS1002</td>
<td>- / NA</td>
<td>- / NA</td>
<td>+ / 3</td>
<td>(1, 3)</td>
</tr>
<tr>
<td>IS6102</td>
<td>+ / 1</td>
<td>- / NA</td>
<td>- / 2.2</td>
<td>(29, 34)</td>
</tr>
</tbody>
</table>

Symbols and abbreviations: +, present in all isolates; (+), present in some isolates; -, absent in all isolates; NA, not applicable; ND, not determined.

• Human-derived B. bronchiseptica isolates only.
• 1 of 13 human-derived isolates was positive (ref #31).
• 4 of 13 human-derived isolates were positive (ref #31).
• Found in rare animal-derived isolates (ref #34).


**Single-Copy Gene Targets**

<table>
<thead>
<tr>
<th>Target</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. pertussis-specific</td>
<td>24</td>
</tr>
<tr>
<td>B. bronchiseptica-specific</td>
<td>24</td>
</tr>
<tr>
<td>Shared among Bordetella species</td>
<td></td>
</tr>
<tr>
<td>Adenylate cyclase</td>
<td>4</td>
</tr>
<tr>
<td>BOP351</td>
<td>9, 26</td>
</tr>
<tr>
<td>Filamentous hemagglutinin</td>
<td>14</td>
</tr>
<tr>
<td>Flagellin</td>
<td>12</td>
</tr>
<tr>
<td>Pertactin</td>
<td>27, 36</td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>11, 22, 31, 32</td>
</tr>
<tr>
<td>PertB</td>
<td>6</td>
</tr>
</tbody>
</table>

With the exception of pertussis toxin, PCR assays targeting these genes have not been well validated.

Species-specific primers, or PCR assays using post-amplification differentiation of species have been described.


**Pertussis Toxin**

- Promoter region ptxP
- Toxin subunit 1A ptxS1/ptxA
- Will detect
  - B. pertussis
  - B. parapertussis
  - B. bronchiseptica (64% of human-derived isolates*)
- Post-amplification analysis (melting peaks) will differentiate species


**Validation Pitfalls—Analytical Sensitivity**

- Can PCR that detects multi-copy IS481 be too sensitive?

**IS481 PCR Crossing Threshold and Clinical Correlation**

IS481 PCR Confirmation by Alternative Methods

<table>
<thead>
<tr>
<th></th>
<th>NH hospital</th>
<th>Mass hospital</th>
<th>Tenn. community</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases by PCR</td>
<td>96</td>
<td>36</td>
<td>48</td>
</tr>
<tr>
<td>Culture</td>
<td>0/27</td>
<td>0/32</td>
<td>1 (index case)/284</td>
</tr>
<tr>
<td>Rpt PCR IS481 and ptxS1 positive</td>
<td>1/111</td>
<td>1/25</td>
<td>-</td>
</tr>
<tr>
<td>Rpt PCR IS481 positive</td>
<td>24/111</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Toxin IgG</td>
<td>-</td>
<td>-</td>
<td>4/11 PCR pos cases</td>
</tr>
</tbody>
</table>

Source: CDC. 2007. MMWR 56:837-42

IS481 and Single Copy Targets Have Similar Diagnostic Sensitivity

<table>
<thead>
<tr>
<th></th>
<th>IS481</th>
<th>ptx promoter</th>
<th>Outer membrane porin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection rate (95% CI)</td>
<td>0.8 (0.75-0.84)</td>
<td>0.76 (0.71-0.81)</td>
<td>0.8 (0.73-0.86)</td>
</tr>
</tbody>
</table>

Pediatric population. 4,442 patients.

Speculated that nonessential IS481 degrades during course of host-pathogen interaction later in disease, decreasing overall sensitivity.


Can IS481 C\textsubscript{T} Value Predict *Bordetella* species?

- *B. pertussis*: >50 copies
- *B. holmesii*: 8-10 copies
- Columbus OH outbreak of pertussis illness, IS481 PCR C\textsubscript{T} values were similar for *B. pertussis* and *B. holmesii* infections (Rodgers et al. CID. 2013)

Real-World Performance of *B. pertussis* PCR

- The good
  - High sensitivity
  - Rapid turn-around time; high throughput
- The bad and ugly
  - During outbreaks, labs often inundated with inappropriate requests for testing
    - Testing asymptomatic persons (exposed? treated)
    - Low pretest probability
  - Co-circulation of other respiratory pathogens

Causes of False-Positives and Pseudo-Outbreaks

- Cross-reactivity of IS481 assay with *B. holmesii*
  - ED nurse
  - Nt sequencing of recA gene confirmed *B. holmesii*

Causes of False-Positives and Pseudo-Outbreaks

- Weakly reactive results from IS481 assay (C\textsubscript{T} values 37-40)
- Solutions
  - Indeterminate range (C\textsubscript{T} >37); confirm using second assay


Causes of False-Positives and Pseudo-Outbreaks

- Environmental contamination in patient clinics
  - Different acellular pertussis vaccines have been shown to contain B. pertussis DNA
  - Aerosolization
  - Contamination of hands, fomites
  - Solutions: vaccinate and collect specimens in different rooms


FDA-Cleared B. pertussis Assay

- Part of the highly multiplexed FilmArray respiratory panel
- Detects toxin promoter region. Melting curve analysis to provide specificity
- Package insert data
  - Sensitivity
    - 4000 cfu/ml (~450 cfu/reaction)
  - Specificity
    - B. bronchiseptica, B. holmesii, B. pertussis not detected up to 10^6 cfu/ml
    - Cross-reactivity at >10^6 cfu/ml
    - During clinical trials, specimens containing B. holmesii were negative

Source: FilmArray Respiratory Panel v1.7 package insert. BioFire Diagnostics

Summary

- IS481-based PCR
  - Limitation that other species will be detected
  - Beware of weak positives. Implement an indeterminate range
- Other insertion elements
  - Multiple targets to distinguish B. pertussis and B. holmesii (e.g. IS481 and hIS1001)
  - Pertussis/parapertussis assay w/o differentiation (IS1002)
- Single-copy targets
  - May require post-amplification analysis (melting curve analysis)
  - B. pertussis-specific targets are not well validated

Summary

- Consider, but don’t fixate on analytical sensitivity. Ultimately, diagnostic sensitivity is most important.
  - Single copy targets such as pertussis toxin likely provide adequate diagnostic sensitivity (no doubt better than culture!)

Summary

- Certain targets may be present in rare isolates of B. bronchiseptica
  - Small number of isolates not sufficient to rule out cross-reactivity
  - Human- and animal-derived isolates may vary re. presence of certain gene targets
- B. holmesii
  - Growing evidence that it has clinical and public health significance, esp. in older patients
  - Too early to group with B. pertussis and B. parapertussis for routine detection

Summary

- ANY QUESTIONS?