Pneumococcal detection and serotyping carriage and invasive specimens

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Lab-confirmed Invasive Disease

Carriage, Mucosal disease

Routine vaccination of infants with PCV7 had profound effects upon the entire iceberg in all ages.
Serotype-specific IPD in children < 5yrs during 2005-2012
Active Bacterial Core surveillance data

In order of decreasing frequency for 2009

National Center for Immunization & Respiratory Diseases
Streptococcus Laboratory/Respiratory Diseases Branch
Collection of Invasive Specimens

- Cerebrospinal fluid, pleural fluid and blood culture collection
- Need good quality specimens
- Aseptic technique
- If possible collect sample before starting antibiotic treatment
- The sample should be transported to the laboratory as soon as possible (ideally < 1 hour)
- Keep the specimen at room temperature (~25C) when in transit
- Use a vaccine carrier with pack of cold water if need to cool the transport box to keep it at ~25C
Bacteriological examination of CSF and pleural fluid

If > 1ml of CSF or PF – freeze 0.5ml in cryovial at -20°C for later PCR

- **CSF:**
  - Binax
  - Centrifuge 0.5ml in sealed tube (2000 rpm for 20 min or 3000 rpm for 15 min)
    - Draw off supernatant and use for latex agglutination (BioRad, Wellcogen etc...)
    - Mix sediment (use for Gram stain, culture on blood, chocolate and MacConkey agar plates) incubate BAP and CAP cultures in CO2 at 37°C for 24h.

- **Pleural Fluid:**
  - Gram stain
  - Binax
  - Cell count
  - Culture (same as CSF)
Blood culture

Requirements:
- Semi-automated with proper pediatric and adult bottles
- Good quality commercial media for BAP, CAP plates with proper blood concentration (good quality brands)
- Timely transport (within 2 hours at room temperature ~25°C)
- 24/7 sub-culture laboratory services
- Proper blood volume (child 1-3ml; adult 5-10ml)
- Contamination rates <5%
- Contamination rates >5% trigger laboratory deep clean-up and phlebotomy procedures refresher training
Blood culture broth (*enrichments)

**REAGENTS**
For *in vitro* diagnostic use.

**CAUTION:** Handle specimens and inoculated culture bottles as though capable of transmitting infectious agents. All inoculated culture bottles, specimen collection needles, and blood-drawing devices should be decontaminated according to your institution’s procedures.²

**BacT/ALERT® SA** (color-coded blue) – BacT/ALERT SA disposable culture bottles contain 40 ml of media and an internal sensor that detects carbon dioxide as an indicator of microbial growth. The media formulation consists of pancreatic digest of casein (1.7% w/v), papaic digest of soybean meal (0.3% w/v), sodium polyanetholesulfonate (0.035% w/v), pyridoxine HCl (0.001% w/v), and other complex amino acid and carbohydrate substrates in purified water. Bottles are prepared with an atmosphere of CO₂ in oxygen under vacuum. The composition of the media may be adjusted to meet specific performance requirements.

**BacT/ALERT® PF** (color-coded yellow) – BacT/ALERT PF disposable culture bottles contain 16 ml of complex media and 4 ml of a charcoal suspension with an average density of 1.0215 g/mL. The media component consists of soybean-casein digest (2.0% w/v), brain heart infusion solids (0.1% w/v), sodium polyanetholesulfonate (0.025% w/v), pyridoxine HCl (0.001% w/v), menadione (0.0000625% w/v), hemin (0.000625% w/v), L-cysteine (0.025% w/v) and other complex amino acid and carbohydrate substrates in purified water. Bottles contain an atmosphere of CO₂ in oxygen and nitrogen under vacuum. The composition of the media may be adjusted to meet specific performance requirements.
**Streptococcus pneumoniae** identification

- **Optochin Disk Test**
  - Streak the suspect alpha-hemolytic colony onto BAP in confluent lines
  - Place 5ug optochin disk with 6mm diameter in the streak inoculum
  - Incubate in CO$_2$-incubator or candle-jar at 35-37°C for 18-24h

  Note: there are *S. pneumoniae* isolates with halo <14 mm Needs ID confirmation with addition of bile solubility test

halo $\geq$ 14 mm *S. pneumoniae*
Streptococcus pneumoniae identification

• Bile Solubility Test
  - Prepare a milky suspension (McFarland No. 1) from an overnight culture in 1 ml of 0.5% saline
  - Divide the suspension in two tubes of 0.5 ml (test and control)
  - Add 0.5 ml of 2% sodium desoxycholate (bile salts) to the test tube and 0.5 ml saline to the control tube.
  - Shake, incubate at 35-37°C for up to 2h (do a first reading after 10 min incubation if negative re-incubate and read after 2 hours)
S. pneumoniae serotyping
Quellung reaction and Multiplex PCR

**Quellung reaction**
- For 91 serotypes
- Costly
- Mostly done in reference labs

**Sequential Multiplex PCR**
- Conventional MPCR for 40 serotypes
- MRT-PCR for 21 serotypes
Conventional multiplex PCR for *S. pneumoniae* serotyping deduction of 40 specificities

Currently includes:

- All conjugated vaccine types: 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F
- The 40 pneumococcal serogroups/serotypes most frequently associated with carriage and invasive disease
- 41 assays (19Fvar – *Menezes et al JCM, 2013*) distributed in 8 reactions that can be designed according to region serotype frequency

Sequential Multiplex PCR for *S. pneumoniae* Serotyping
(http://www.cdc.gov/ncidod/biotech/strep/pcr.htm)

- **Latin American set**

  - **Reaction 1**
    - Serotypes 6, 14, 9V, 19A, 23F
    - Positive only for *cps* locus

  - **Reaction 2**
    - Serotypes 3, 15B, 17F, 18, 19F
    - Positive only for *cps* locus

  ~80% invasive serotypes in Latin America

  ![Reaction 1](14 6 23F 19A 9V)
  ![Reaction 2](19F 3 15B 18 17F)
Conventional multiplex PCR *S. pneumoniae* serotype deduction

**Controls**

- Non template controls
  - Reagent control – blank (water)
- Positive template serotypes controls
  - Validate reaction performance
- *S. pneumoniae* capsule control
  - *cpsA* easily detected for most pneumococcal isolates
    - *cpsA* primers frequently fail to amplify for serotypes: 38 and 25F; more rarely for 3, 14 and 35A
Potential problems with conventional multiplex PCR assays

• False Positives
  ▪ Non-specific amplification of primers
  ▪ Contamination of template material

• False Negatives
  ▪ Inhibition of PCR reaction
  ▪ Problems with the master mix reagents
  ▪ Failure of nucleic acid extraction procedure
  ▪ Failure to add template (sample)
Conventional multiplex PCR assay results

Analysis of fragments sizes

- The size of the amplified fragment should match with the positive control of specific serotype (check with the ladder).

- It is frequent to find clinical samples such as pleural fluid with non-specific amplification that needs to be confirmed by monoplex PCR for the closest size target.

- Non-specific DNA amplification most likely produce a fragment with different size from the ones produced by the serotype control.

- Recently a significant amount of lytA-negative (non-pneumococcal) isolates from carriage specimens have shown amplification for the serotyping assays: 10F/10C/33C, 18C/18B/18A/18F, 5, 12F/12A/12B/44/46, 13 and 15A/15F (Carvalho et al. PeerJ 2013).
Real-time PCR for *Streptococcus pneumoniae* serotyping (only for *lytA* positive samples)

- 21 serogroup/serotypes in 7 triplex reactions:

**Latin America set:**
- reaction 1 (serotypes: 14, 18C/18F/18B/18A, 19F)
- reaction 2 (serotypes: 4, 6A/6B/6C/6D, 9V/9A)
- reaction 3 (serotypes: 1, 5, 23F)
- reaction 4 (serotypes: 3, 7F/7A, 19A)
- reaction 5 (serotypes: 6C/6D, 12F/12A/12B/44/46, 22F/22A)
- reaction 6 (serotypes: 15A/15F, 23A, 33F/33A/37)
- reaction 7 (serotypes: 2, 11A/11D, 16F)

*Pimenta et al. JCM 2013*

- **reaction 8 (serotypes: 19Fvar, 15B/15C, 10A)**
High sensitivity of real-time PCR increases the chances for detection of cross-contamination

**Optimal set-up:**

- Separate rooms with hoods for reaction assembly (clean room) and DNA template addition (dirty room)*
- Hoods with UV capability
- Use a different clean lab-coat in each of the rooms
- Change gloves frequently
- Use a separate set of pipettors in each room
- Always use filtered barrier pipette tips

* At minimum, use separate benches and micropipettes for template preparation and reaction assembly
Data Interpretation

The following cutoff values are used in the *Streptococcus Laboratory*

- **Positive** = $C_t \leq 35$
- **Negative** = $C_t > 40$
- **Equivocal** = $C_t$ 36 - 40

Equivocal results may be due to low yield of DNA or PCR inhibitors present in clinical specimens such as heme (in blood hemoglobin), salt, enzymes, nucleases, etc.

Re-test equivocal specimens undiluted in triplicate and diluted 1:5 and 1:10 in water to dilute potential inhibitors. If any have a $C_t$ value $\leq 35$, consider the sample positive.
Potential challenges and suggestions for real-time PCR assay

• False Positives
  – Non-specific hybridization of primers and probe
  – Non-specific probe hydrolysis
  – Contamination of template material

• False Negatives
  – Failure of nucleic acid extraction procedure
  – Failure to add template (sample)
  – Inhibition of PCR reaction
  – Incomplete master mix

\[\text{Endogenous Control (RNAseP gene assay)}\]
\[\text{Internal Positive Control}\]
Potential challenges and suggestions for real-time PCR assay

High or no Ct counts in known positives samples

- Was the correct DNA extraction process performed?
  - If pre-treatment with mutanolysin and lysozyme are not used, DNA from gram positive bacteria may not be efficiently extracted and thus will not be detected

- Are PCR inhibitors present?
  - Template DNA can be diluted 1:5, 1:10 and retested
  - Can test for human specific \( RNAseP \) gene

- Were all reaction components added?

- Were specimens handled properly? Was the specimens volume sufficient?
  - Clinical specimens should ideally be shipped on dry ice and frozen at -20°C or -80°C. The sample volume for extraction should be at least 200 ul for optimal yields
Potential challenges and suggestions for real-time PCR assay

Ct counts in known negative controls usually means cross-contamination

- Clean rooms and dirty rooms with separate sets of pipettes should be used
- Lab-coats and gloves should be changed between rooms
- 0.5-1.0% bleach and 70% ethanol should be used to spray work areas
- Consumable reagents should not be re-used
- Reaction tubes should be protected during transport from clean room to dirty room with caps
- The amount of positive control DNA used should be limited (Ct ~20-25)
- Reaction tubes should not be re-opened and should be immediately discarded
### TaqMan Template - *S. pneumoniae* serotypes

#### Real-time reaction for serotyping

<table>
<thead>
<tr>
<th>Component</th>
<th>1Rx (µl)</th>
<th>(20) Rx (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invitrogen-Platinum Quantitative PCR SuperMix-UDG</td>
<td>12.5</td>
<td>250</td>
</tr>
<tr>
<td>PCR H2O</td>
<td>vary</td>
<td>80</td>
</tr>
<tr>
<td>MgCl2 (50uM)</td>
<td>1.5</td>
<td>30</td>
</tr>
<tr>
<td><strong>ROX (25uM, diluted 1:10)</strong></td>
<td>0.5</td>
<td>10</td>
</tr>
<tr>
<td>Primers-F (10uM)</td>
<td>vary</td>
<td>10</td>
</tr>
<tr>
<td>Primers-R (10uM)</td>
<td>vary</td>
<td>10</td>
</tr>
<tr>
<td>Probes-FAM/HEX/CY5 (10uM)</td>
<td>vary</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>20.0</td>
<td>400</td>
</tr>
<tr>
<td>DNA sample</td>
<td>5.0</td>
<td></td>
</tr>
</tbody>
</table>

#### Thermal Cycling Parameters

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X</td>
<td>95°C</td>
<td>10 min</td>
</tr>
<tr>
<td>40X</td>
<td>95°C</td>
<td>15 sec</td>
</tr>
<tr>
<td></td>
<td>60°C</td>
<td>1 min</td>
</tr>
</tbody>
</table>

**Methods for Spn detection and serotyping from carriage**

1. **In the field**
   - NP swab collected and vortexed
   - Transport in 1.0 ml STGG medium at 4-6 hours, stored at -70°C

2. **In the lab**
   - Quick thaw and vortex
   - Return to store at -70°C
   - 200ul NP-STGG
   - 5.0 ml enriched broth pre-culture
     (Todd Hewitt w/0.5% yeast + 1ml rabbit serum)
   - 6h/37°C, 5%CO2
   - 10ul

   Culture isolation on BAP:
   - 1 colony per morphology
   - Identification (optochin and bile solubility)
   - PCR - Quellung for serotyping
Preparation of skim milk, tryptone, glucose, glycerol transport/storage medium (STGG)

STGG formula:
- Skim milk powder (from Difco or grocery) 2 g
- Tryptone soya broth (TSB, from Oxoid) 3 g
- Glucose 0.5 g
- Glycerol 10 ml
- Distilled water 100 ml

- Mix to dissolve all ingredients.
- Dispense in 1.0 ml amounts in screw-capped 1.5-ml vials.
- Loosen the screw-cap tops and autoclave for 10 minutes (at 115 °C). Tighten caps after autoclaving.
- Store STGG frozen at -20°C or refrigerate until use. Use STGG medium within 6 months of preparation.

### Results: Urban Kibera Children < 5 years

<table>
<thead>
<tr>
<th>Year</th>
<th>Number Enrolled</th>
<th>No. (%) Spn carriage</th>
<th>No. (%) PCV10 carriage among Spn carriers</th>
<th>% PCV10 carriage among all participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009 (baseline #1)</td>
<td>545</td>
<td>488 (90)</td>
<td>206 (42)</td>
<td>38</td>
</tr>
<tr>
<td>2010 (baseline #2)</td>
<td>258</td>
<td>233 (90)</td>
<td>98 (42)</td>
<td>38</td>
</tr>
<tr>
<td>2011 (PCV10 introduction)</td>
<td>408</td>
<td>362 (89)</td>
<td>122 (34)</td>
<td>30</td>
</tr>
<tr>
<td>2012 (post-introduction #1)</td>
<td>309</td>
<td>284 (92)</td>
<td>53 (19)</td>
<td>17</td>
</tr>
</tbody>
</table>

Spn = *Streptococcus pneumoniae*
Conclusions

- Preliminary results show a decrease in PCV10-type carriage among Kibera children
  - PCV10 is making an impact in Kenya
Gracias!
Todos están bienvenidos a la Copa
Y viva a Neymar

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Streptococcus Laboratory-CDC/NCIRD/DBD/RDB
http://www.cdc.gov/nciod/biotech/strep/protocols.htm