Multiplex PCR Approach for Respiratory Pathogens

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Presentation

- Background
- Needs for multiplex approach
- Technologies and kits available
- Experience in our Lab
- Validation in EN 17045 context
- Pittfals
- Conclusion
Emergences

- 2001  Human Metapneumovirus
- 2003  SARS - HCoV
- 2005  Coronavirus NL63, HkU1 ; Human Bocavirus
- 2007  Rhinovirus type C
- 2009  H1N1 2009
- 2009  H5N1 - H7N9
- 2012  MERS - HCoV
Epidemics

- Seasonal: RSV, influenza A, metapneumovirus
- Sporadic and clusters: SARS
- Pandemia: H1N1 2009 influenza A
- Alerting threat: H7N9 or H5N1 influenza A
- Recently: MERS-HCoV

➤ Surveillance: OMS, CDC, ECDC, BAG or at local level
Which Pathogens?

- Clinically significant
- Patients CAP, nosocomial, immunocompromised
- Treatment
- Hospital Infection Control Unit, Isolation
- Epidemiology
- Emergent pathogens
- Prevalence in the Laboratory
Clinical point of view

- Severe Pneumonia
- Atypical pneumonia
- Acute Respiratory distress
- Chronic diseases
- Immunocompromised or suppressed patient
- Newborn
### Background: GRACE Study

<table>
<thead>
<tr>
<th>Aetiology of LRT Infection (n=3059)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>9.1</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td>14.8</td>
</tr>
<tr>
<td><em>Mycoplasma pneumoniae</em></td>
<td>2.9</td>
</tr>
<tr>
<td><em>Chlamydia pneumoniae</em></td>
<td>2.2</td>
</tr>
<tr>
<td>Virus</td>
<td>51.1</td>
</tr>
</tbody>
</table>
Direct Diagnostic

- Culture
  - for mainly classical bacterial pathogens
  - Semi-quantification
  - Antibiogram
  - Virus?

- Antigen detection
  - Virus (RSV, influenza A)
  - *Legionella pneumophila* type 1
  - *S.pneumoniae*
  - Virus panel in IF
## Significant pathogens: the others

<table>
<thead>
<tr>
<th>First line</th>
<th>Second line</th>
</tr>
</thead>
<tbody>
<tr>
<td>INFLUENZA</td>
<td>METAPNEUMOVIRUS</td>
</tr>
<tr>
<td>PARAINFLUENZA</td>
<td>RHINOVIRUS</td>
</tr>
<tr>
<td>RSV</td>
<td>ENTEROVIRUS</td>
</tr>
<tr>
<td>ADENOVIRUS</td>
<td>CORONAVIRUS</td>
</tr>
<tr>
<td>H1N1 - INF B - ?</td>
<td>BOCAVIRUS</td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>Chlamydia pneumoniae</td>
</tr>
<tr>
<td>Bordetella pertussis</td>
<td>Bordetella parapertussis</td>
</tr>
<tr>
<td></td>
<td>Legionella pneumophila</td>
</tr>
</tbody>
</table>

Adapted from Ieven M; ECCMID 2012
Impact of Molecular Diagnostic

- 42% more virus detected
- Sensitivity of 98.5% for molecular vs of 68.8% for culture and antigenic tests. (Mahony J et al. J. Clin. Microbiol. 2007)
- Aetiology improved in children: 24% to 43%
- Aetiology improved in adults: 3.5% to 36%
- INCREASE Identification of Pathogens
- INCREASE aetiology recognition
First experience
Poster at RICAI 2011

- Study during 2008 - 2010
- Children from <9 years old
- Gr1: Bronchiolitis + positive RSV antigen
- Gr2: Bronchiolitis + negative RSV antigen
- Gr3: Suspicion of H1N1 2009
- CLART® Pneumovir V3
## Results

<table>
<thead>
<tr>
<th></th>
<th>RSV PCR</th>
<th>PCR NEG</th>
<th>OTHER</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV ICT+</td>
<td>19</td>
<td>2</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>RSV ICT -</td>
<td>11</td>
<td>13</td>
<td>41</td>
<td>65</td>
</tr>
<tr>
<td>H1N1 -</td>
<td>0</td>
<td>11</td>
<td>24</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>36</td>
<td>69</td>
<td>125</td>
</tr>
</tbody>
</table>

- 37% RSV missed by ICT
- 20% RSV ICT negative were actually PCR negative
- 68.6% H1N1 2009 PCR negative show other viruses
- « Thinking RSV » and missing many other virus!
Tech Needs or Tech Geeks

- What do we need actually?
- The newest the better
- More sensitivity
- Larger Panel
- Easy identification
- All-in One
- Higher income
Diagnostic Needs

- Up to date Microbiology Lab
- Rapid Diagnostic
- Exhaustive Panel of Pathogens

Or maybe more efficiently:

- Constructed clinical diagnostic
- Dialogue between Clinician and the Microbiologist

Why not all included?
Available Assays

- CLART Pneumovir (Genomica)
- RespiFinder (Pathofinder)
- xTAG RVP (Luminex)
- Aniplex RV16 (Seegene)
- Xpert FLU (Cepheid)
- Resp.Panel FilmArray (BioFire)
- Puzzle Multiplex or Self Choice PCR (TIB; Fast-Track)
Value of Test

- Sensitivity – specificity
- Predictive Values (PPV, NPV) in their adequate prevalence situation
- Likelihood Ratios (LR+, LR-)
- Field of Application
- Pitfalls
Aniplex II vs Pneumovir

- 24 EQC samples (QCMD 2012 for influenza, rhinovirus, adenovirus)
- 34 prospective samples from pediatric ward (HNE)
- 15 prospective samples asking for the Respiratory Panel
- Dec2012 to Feb2013
- Two step amplification real time detection versus end point PCR with a microarray detection
- Both test executed within a day
Aniplex II vs Pneumovir

<table>
<thead>
<tr>
<th></th>
<th>PNEUMOVIR</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>54</td>
<td>7</td>
<td>61</td>
</tr>
<tr>
<td>Negative</td>
<td>4</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>12</td>
<td>70</td>
</tr>
</tbody>
</table>

Kappa = 0.386
Pneumovir vs Aniplex II

- No correlation; 15%
- 1 virus at least; 19%
- Virus different; 4%
- All virus; 62%
Third Assay

- Third amplification based on nested PCR
- Realtime detection
- Completely closed and automated from extraction to final detection.
- 21 pathogens in 65 minutes
FilmArray: Nested PCR

Sample extraction & purification

1st stage multiplex PCR

2nd stage PCR

Reagent Storage
Steps

1. Hydration Solution
   Transfer to the pouch
   Vacuum aspiration (1ml)

2. Sample in Lysis Buffer
   Transfer to the pouch
   Vacuum aspiration

3. Transfer to the cycler
FilmArray

- PCR2 in real time
- Multiple singleplex
- Intercalant fluo dye
- Melting Curves
- RNA Process Control
- PCR2 Control
FilmArray (BioFire)

- Routine first samples March to May 2013
- Original requests: « Flu » - « wheezing » - « whooping cough » - « respiratory panel »
- Age from 6 months to 91 years old
- Classical Respiratory viruses Panel
- Used also as third detection kit to look at results showing no agreement between AniplexII and Pneumovir
## Results

<table>
<thead>
<tr>
<th></th>
<th>Nég</th>
<th>Infl. A</th>
<th>Infl. B</th>
<th>PIV1</th>
<th>PIV2</th>
<th>PIV3</th>
<th>Adéno</th>
<th>MPV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>39</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>RSV</th>
<th>HKU1</th>
<th>NL63</th>
<th>229E</th>
<th>OC43</th>
<th>Boca</th>
<th>RV/EV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>B.pertu</th>
<th>M.pne</th>
<th>C.pne</th>
<th>QCMD</th>
<th>M.pne</th>
<th>C.pne</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>9/12</td>
<td>11/12</td>
<td></td>
</tr>
</tbody>
</table>

Total of 70 samples, 44.3% were positive
3 double infections (4.3%)
QCMD Core 100% for M.pneumoniae and C.pneumoniae
Discussion

- One panel = 100 % correlation
- Two panels = 66% ???
- Three panels <50% ?!
- The more, the worst - so what shall we do with those panels?
- Proposal would be to use them as screening test and use a quantitative monoplex to correlate with the clinic

» Multiplex are less sensitive than singleplex!
Sensitivity versus monoplex

<table>
<thead>
<tr>
<th>Virus</th>
<th>RespiFinder</th>
<th>X TAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influenza</td>
<td>84.8 %</td>
<td>72.3 %</td>
</tr>
<tr>
<td>Corona</td>
<td>89.1 %</td>
<td>32.6 %</td>
</tr>
<tr>
<td>Metapneumo</td>
<td>100 %</td>
<td>96.6 %</td>
</tr>
<tr>
<td>Rhino</td>
<td>95.2 %</td>
<td>88.7 %</td>
</tr>
<tr>
<td>RSV</td>
<td>88.5 %</td>
<td>71.4 %</td>
</tr>
</tbody>
</table>

Both assays are less sensitive than in-house PCR

GRACE study 2012 (Ieven, ECCMID Berlin 2013)
Validation : What shall we do ?

- LOOK FOR THE Assay’s Values !
- Complete with A VALIDATION
  - Home made Assays
  - Puzzle kit Assays
  - Whole kit Assays
  - POCT PCR
  - Research Assays RUO, CE-IVD, FDA
Validation:
What has already been done

- Research Assays, CE, FDA
- Publications (company or independent studies)
- Reactive products from different producer put together
- Certification ISO 9000 Company
Validation: How extensive?

- Samples
- Transport media and Reagents
- Reference material (n=?, species, types, variants)
- Manipulation, internal procedure
- How many tests to qualify? Routine samples (n=?)
- How much to Accreditate (ISO 17025, ISO/EN 15189)

➢ No consensus so far!

REFLECTIONS AND PROPOSALS TO ASSURE QUALITY IN MOLECULAR DIAGNOSTICS. MolecularDiagnostics.be working group (Acta Clinica Belgica, 2011; 66-1)
Quality Control

- EQC for each parameter?
- EQC Panel availability?
- IQC Panel to control lot?
- IQC Technical qualification?
- Test Validation with positive sample for each parameter?
- Test Validation with negative sample
About commercial Panel

- Pathogen included: how good is the choice?
- How fast is it adapted to genetic changes?
- How good is sensitivity?
- How sure is specificity?
- Amount of waste (Test, package, aluminium, toxicity)
- High Costs (?)
Pitfall 1

- FilmArray RP Panel M210 v1.1 (Maine Molecular Quality Controls, Inc)
- Pair of tubes containing all the positive and negative IQC included in assay
- Results all negative although the control passed
  - How SECURE are individual results?
  - How reliable the procedure?
Pitfall 2

- Retro-nasal sample from a patient presenting cough since 2 months
- FilmArray is negative
- Rapid serological test for M.pneumoniae positive
- Monoplex PCR show positive at 53c/ml (!)

- How sensitive we need the test to be?
- Are some rapid test more efficient?
Conclusion

- Multiplexing PCR Test is a way to open to new aspect of clinical signs and disease by identifying etiology
- Validation – is an essential issue to discuss
- Sensitivity should correlate with clinical criteria
- Prevalence of pathogens in none clinically ill cases
- Emergence, epidemiology, are challenging; assay has to be updated and keep track of genomic changes.
- Dialogue to bind kit characteristics, limits, and pitfalls to the clinical aspect
Final: Panel Concept

- All-in-One
  - Pneumovir – Aniplex - RespiFinder – xTAG – POCT - FilmArray

- Ones-in-Whole
  - or lots of monoplex and choose the one you need and multiply the test number
Vision : Diagnostic Lab 2020

Thank you for your attention