Molecular Diagnostics at Point of Care

When will we get there; and where is ‘there’ anyway?

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Learning Objectives

- Participants should be able to:
  - Describe the basic work-flow of molecular diagnostic testing.
  - Describe some major amplification and detection methods.
  - Recognize the properties of analytes that make them candidates for molecular testing.
  - Recognize emerging molecular diagnostic platforms that may be usable at point-of-care.
  - Assess platforms for influenza testing in the context of POCT.
  - Describe unique quality issues in molecular diagnostics which impact their use at point of care.
  - Recognize Campbell’s Laws of POCT and their implications for the future of molecular methods.
What is Molecular Diagnostics?

- Analysis of DNA or RNA for diagnostic purposes. Molecular diagnostics have found widespread application with the advent of *amplification methods* (PCR and related approaches).

- Huge scope
  - From single-target molecular detection of pathogens...
  - To pharmacogenomic analysis of metabolism genes for drug dosing...
  - To whole genome sequencing for disease susceptibility and God knows whatall.
Molecular Diagnostic Testing

- Specimen
- DNA / RNA Extraction
- Amplification of Target
- Detection of amplified target
- Interpretation and Clinical Use

Poll questions 1-3
Why Amplify?

- **Sensitivity**
  - can detect small numbers of organisms
  - can even detect dead or damaged organisms
  - can detect unculturable organisms

- **Speed**
  - 4-48 hour turnaround
  - inoculum independence
Why Amplify, continued

- **Targets**
  - Test for things there’s no other way to test
  - Uncultivable bugs

- **Genetics**
  - Pharmacogenomics
  - Prenatal testing
  - Hypercoagulability, etc.

- **Oncology**
  - Hematologic malignancies
    - Diagnostic markers
    - Minimal residual disease
Why Not Amplify?

- Clinical significance?
- Technical problems
  - Contamination
  - Inhibition
- Cost
- COST
- CO$T
Extraction

DNA/RNA Extraction

- Depends on:
  - Specimen source (blood, CSF, stool)
  - Target organism (human tumor, CMV, M. tuberculosis)
  - Target nucleic acid (DNA, RNA)

- Increasing automation
  - Magnetic or other separation methods.
  - REQUIRED for POC
Amplification

- Nucleic Acid Amplification means taking a small number of targets and copying a specific region many, many times.
- NAAT, NAT, etc; commonly-used abbreviations
- PCR is the most common amplification scheme, but there are others!
Amplification Enzymology

- **DNA polymerase**
  - makes DNA from ssDNA, requires priming

- **RNA polymerase**
  - makes RNA from dsDNA, requires specific start site

- **Reverse transcriptase**
  - makes DNA from RNA, requires priming

- **Restriction endonucleases**
  - cut DNA in a sequence specific manner

Lots!
Polymerase Chain Reaction (PCR)

Target DNA + Primer oligonucleotides (present in excess)

Split DNA strands (95°C 5 min), then allow primers to bind (40-70°C)

DNA polymerase extends the primers (40-80°C) to produce two new double-stranded molecules

Repeat the split-bind-extend cycle

This ‘short product’ amplifies exponentially in subsequent split-bind-extend cycles, driven by the temperature changes in a ‘thermal cycler’.
Reverse Transcriptase PCR (RT-PCR)

Target RNA + Primer oligonucleotide

Primer binding (RT - 37°C)

Reverse Transcriptase (RT) makes a DNA copy of the RNA target

The DNA copy is used in a PCR reaction
Other Amplification Methods

- PCR isn’t all there is!
  - Transcription-mediated amplification (TMA)
  - Loop-mediated isothermal AMPlification (LAMP)
- Others
  - Isothermal technologies decrease the complexity of the instrument required.
Detecting PCR Products in the Old Days

- Gel electrophoresis (± Southern blotting)
- Enzyme-linked assays
- Hybridization
  Protection/chemiluminescent assay
- A multitude of formats available, to serve market and technical needs

- Specimen
- DNA / RNA Extraction
- Amplification of Target
  Detection of amplified target
- Interpretation and Clinical Use
Real-Time PCR

- Combination
  - Detection
  - Amplification
- RT-PCR Instruments monitor product formation by detecting change in fluorescence in a tube or well during thermal cycling.
- Frequently use PCR for amplification
  - Robust
  - Off-patent

Figure 2. Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR (Figure from Applied Biosystems’ DNA/RNA Real-Time Quantitative PCR bulletin).
Real-Time PCR Instruments

- Contain three functional components
  - A thermal cycler
    - Mostly a single cycler that cycles all the tubes / wells at the same time
    - The SmartCycler and GeneExpert have individually controllable cycler elements.
  - Fluorescent detection system
    - The number of fluorescent detection channels determines how many different probes you can use.
    - An internal amplification control is a must.
  - A computer to run the components, interpret the data, etc.
Real-time PCR Chemistries

- Essential Fluorescence Chemistry
  - Shorter wavelength = higher energy
  - Activation with high-energy light, usually UV
  - Emission at a lower energy, usually visible
  - Different fluorochromes have different (and hopefully distinguishable) activation and emission wavelengths.

- The more fluorochromes a real-time instrument can detect, the more ‘channels’ it is described as having, and the more targets can be detected.
Quenching

- Fluorescence occurs when a photon bumps an electron to a higher energy level, then another photon is emitted when it drops back to ground state.
- Some compounds (‘quenchers’) suck up that energy before it can be reemitted, ‘quenching’ the fluorescence.
- This is distance dependant; the closer the molecules are the more efficient the quenching.
Fluorescence Resonance Energy Transfer (FRET)

- A second fluorochrome can suck up the energy from the activated fluorochrome and re-emit it at its emission frequency.

- This is distance dependant; the closer the molecules are the more efficient the energy transfer.
Real-Time Detection Schemes

- Taqman Probes
- FRET Probes
- Molecular Beacons
- Several others
Contamination!

- **What happens** when you make $10^6$ copies of a single short sequence in a 100ml reaction?
  - You end up with $10^4$ copies/ul
  - What happens when you pop the top off a microcentrifuge tube?
    - ...or pipet anything
    - ...or vortex anything
    - ...or...

- **You create aerosols**
  - Droplet nuclei with diameters from 1-10 $\mu$m persist for hours/days
  - Each droplet nucleus contains amplified DNA
  - Each amplified molecule can initiate a new amplification reaction
Ways to Prevent Contamination

- Meticulous technique
  - Hoods, UV, bleach, physical separation of work areas

- Assay design
  - Avoid opening tubes for reagent addition, etc.
  - Reactions that produce RNA products
  - Negative controls
  - Real-time assays with closed-tube detection

- Chemical and Physical Inactivation
POC Molecular Diagnostics

- Infectious Disease
  - Outpatient POC
    - GC / Chlamydia
    - Group A strep
    - HIV / HCV viral load
    - GI pathogens
  - Acute-care POC – Lab vs POC
    - Respiratory pathogens
    - CNS pathogens
  - Nosocomial / Screening
    - MRSA / VRE
    - C. difficile
  - Biopreparedness
    - Military development and applications
  - Diseases of Under-resourced populations
    - Tuberculosis incl drug-resistance

- Others
  - Pharmacogenetics
  - Hypercoagulability
  - Other genetic diseases
  - Oncology
    - Lower priority for POC
    - Large number of diseases
    - Solid tumors – need tissue
    - Generally easier follow-up.

- NOTE: the ones in pink actually exist in some FDA-approved form of moderate complexity or waived. The rest are in active development.
What’s First?

- Things that’re easy
  - MRSA, already on GeneExpert (arguably the first simple molecular platform)
- Things that’re hot
  - Influenza and other respiratory viruses
- Things where existing tests perform poorly
  - Respiratory viruses in general
  - Group A strep
  - Group B strep
- Things for hard-to-reach populations
  - *Chlamydia* and gonorrhoea
  - Tuberculosis and other diseases in poor parts of the world.
What Will a Molecular POC Test Look Like?

- Automated, fully integrated
  - Sample preparation
  - Amplification and detection
  - Reproducibility
  - Reliability
  - Such systems are emerging

- Quality need not be compromised for POC molecular tests
  - Unlike most of the antigen tests versus lab-based methods
### Why Molecular? Rapid flu versus Other Methods

<table>
<thead>
<tr>
<th>Rapid Test</th>
<th>Sens%</th>
<th>Spec%</th>
<th>Compared With</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>3M QuickVue</td>
<td>75%</td>
<td>98%</td>
<td>Culture</td>
<td>Archived specimens</td>
<td>Dale et al JCM 46(11):3804-7, 2008 Nov</td>
</tr>
<tr>
<td>BinaxNow</td>
<td>53%</td>
<td>100%</td>
<td>RT-PCR</td>
<td>2 of 237 samples were flu B pos by RT-PCR but flu A by NOW</td>
<td>Landry et al JCV. 43(2):148-51, 2008 Oct</td>
</tr>
<tr>
<td>BinaxNow</td>
<td>61%</td>
<td>100%</td>
<td>RT-PCR</td>
<td>DFA was 81% sensitive</td>
<td>Rahman et al Diag Micro Infect Dis 62(2):162-6, 2008 Oct</td>
</tr>
<tr>
<td>RemelSpect</td>
<td>47.7%</td>
<td>98.7%</td>
<td>Culture</td>
<td>20.3/99.8 Flu B / 35.9/99.9 Flu B</td>
<td>Cruz et al JCV 41(2):143-7, 2008 Feb</td>
</tr>
<tr>
<td>BinaxNow</td>
<td>52%</td>
<td>100%</td>
<td>RT-PCR</td>
<td>70% in days 1-3 of disease</td>
<td>Nilsson et al Inf Cont &amp; Hosp Epi 29(2):177-9, 2008 Feb</td>
</tr>
<tr>
<td>Directigen</td>
<td>42%</td>
<td>96%</td>
<td>Culture</td>
<td></td>
<td>Rahman et al Diag Micro Infect Dis 58(4):413-8, 2007 Aug</td>
</tr>
<tr>
<td>BinaxNow</td>
<td>73%</td>
<td>99%</td>
<td>RT-PCR</td>
<td>Sensitivity only 30% vs flu B for all</td>
<td>Hurt et al JCV 39(2):132-5, 2007 Jun</td>
</tr>
<tr>
<td>Directigen</td>
<td>63%</td>
<td>97%</td>
<td>RT-PCR</td>
<td>Data pooled from all rapids</td>
<td>Mehlmann et al JCM 45(4):1234-7, 2007 Apr</td>
</tr>
</tbody>
</table>

Convenience sample of recent literature; selected by Medline search + fit to single page
Molecular Testing for Influenza

- Real-time methods can provide result in <1h.
- Molecular methods as a class exceed culture in sensitivity (probably due to viral loss in transport)
- Detection properties do vary from system to system – do your homework!
- Moderately to very expensive equipment
- Multiple methods of waived to high complexity.
- Now clearly the ‘gold standard’

Information sources:

- CAP Website for some price information
- Manufacturer’s web sites and PubMed for pictures, workflow and other information.
FDA-approved Molecular Influenza Tests

- **Waived complexity**
  - Alere i Influenza A and B
  - Roche LIAT Influenza A/B Assay

- **Moderate or High complexity**.
  - Cepheid Xpert Flu Assay
  - eSensor Respiratory Viral Panel
  - FilmArray Respiratory Panel
  - Prodesse PROFLU and PROFAST
  - Quidel Molecular Influenza A+B Assay
  - Qiagen Artus Influenza A/B Rotor-gene RT-PCR kit
  - Simplexa Flu A/B & RSV and Flu A/B & RSV Direct and Influenza A H1N1 (2009)
  - Verigene Respiratory Virus Nucleic Acid Test and RV+ Test
  - X-TAG Respiratory Viral Panel and RVP-FAST
Alere I Influenza A&B

- CLIA-waived
  - Bring supplies to room temperature.
  - Put test base and sample receiver on instrument; allow to warm.
  - Place swab in sample receiver, mix.
  - Apply transfer cartridge to sample receiver.
  - Move transfer cartridge to test base.
  - Close lid; test runs 10 minutes.
Roche LIAT Influenza A/B Assay

- CLIA waived
- LIAT stands for Lab-In-A-Tube
- Detects Influenza A&B
- Sample to answer .5h
Cepheid Xpert Flu Assay

- Moderately complex
- Detects Flu A and B; discriminates 2009 H1N1.
- Flu + RSV cartridge available
- Sample to answer ~1h
- GeneXpert Xpress waived in 12/2015
FilmArray Respiratory Panel

- Moderately complex
  - Working toward waived
  - From: Biofire (BioMerieux)
- Detects: Influenza A and B (discriminates H1, H3, 2009 H1)
  - Respiratory Syncytial Virus,
  - Parainfluenza 1, 2, 3 and 4 virus,
  - Human Metapneumovirus,
  - Rhinovirus/Enterovirus,
  - Adenovirus, 4 Coronavirus variants,
  - Bordetella pertussis,
  - Mycoplasma pneumoniae,
  - and Chlamydophila pneumoniae
- Sample to answer ~1h
Simplexa Flu A/B & RSV and Flu A/B & RSV Direct and Influenza A H1N1 (2009)

- Highly complex (Direct version is Moderately complex)
- From Focus Diagnostics / 3M
- Detects Influenza A&B and RSV; a separate test discriminates 2009 H1N1
- Sample to answer ~4h, ~2h for Direct
Verigene Respiratory Virus Nucleic Acid Test and RV+ Test

- Moderately complex
- From Nanosphere
- Detects Influenza A & B, RSV A&B, Plus version discriminates H1, H3, and 2009 H1N1
- Approved for NP swabs
- Sample to answer 3.5h
Are All Molecular Tests The Same?

- Of course not. That would be too simple.
- Numerous, rather confusing studies.
  - There are few comparisons of multiple methods. Sorry.
  - Don’t take this as a comprehensive assessment of both assays; neither performed as well as the authors’ homebrew RT-PCR.
- Performance DOES vary within the molecular tests.
- Pay attention not only to sensitivity / specificity numbers, but also to comparator method.
  - Comparisons with culture make a method look better; comparisons with a highly optimized molecular method or with a panel of different methods is a more stringent comparison.

<p>| Table 1 |
| Sensitivity of the Verigene RV+ test and the Simplexa Flu A/B &amp; RSV kit by virus (n = 350) |</p>
<table>
<thead>
<tr>
<th>Test</th>
<th>% Sensitivity for&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Influenza A virus</td>
</tr>
<tr>
<td>Verigene RV+</td>
<td>96.6 (56/58)</td>
</tr>
<tr>
<td>Simplexa</td>
<td>82.8 (48/58)</td>
</tr>
</tbody>
</table>

Speed and Multiplexing and Complexity

![Graph showing Speed and Multiplexing and Complexity]

- **Waived**:
  - FilmArray
- **Moderately / Highly Complex**:
  - XTAG RVP
  - eSensor RVP
  - XTAG RVP FAST

- Time to result (hr):
  - 0
  - 2
  - 4
  - 6
  - 8

- # of targets:
  - 0
  - 2
  - 4
  - 6
  - 8

- Products:
  - Alere I Influenza A/B
  - Cepheid Xpress Flu/RSV
  - Roche LIAT
  - Alele I Influenza A/B
  - Simplexa Direct
  - Qiagen Artus
  - Simplexa Quidel Flu
Does it Make Sense to Test?

- Cost-effectiveness studies are tricky.
- Assuming a $50,000 per quality-adjusted life-year willingness-to-pay threshold, the most cost-effective treatment option is treatment according to provider judgment from 0% to 3% prevalence, treatment according to a PCR-based rapid influenza test from 3% to 7% prevalence, and treating all at greater than 7% prevalence.
  - …but this ignored induction of antiviral resistance, transmission of flu, and cost avoidance in tested patients; only treatment cost and effect was counted.
  - “Patients who did not have influenza were not evaluated further because influenza testing or treatment would have no further effect on their care or outcomes.”
When to test?

- Remember – false-positives have potentially severe consequences, e.g. non-treatment of a serious bacterial infection.
- Test during the flu season.
  - This is the conventional wisdom, to be modified in travelers and people with contacts who are travelers. Note that other viruses don’t have influenza’s striking seasonality.
  - Molecular tests may have higher specificity than the old antigen tests, but still; question off-season positives.
- Potential strategies:
  - Seasonal: test Oct-Dec→March or so.
    - Early season – retain specimen for confirmatory testing!
  - Incidence-based testing – monitor regional influenza per CDC and State systems, begin testing only when influenza reported in the area.
- Remind providers to test *early in illness*, the best therapeutic results are when drugs are started within 48h of onset.
Who to Test?

- Expensive molecular flu tests may be best deployed selectively.

- Consider testing:
  - Patients destined for hospital admission.
  - Compromised patients at high risk likely to benefit from treatment.

- Consider not testing:
  - Otherwise healthy people who probably don’t need anything but reassurance and good hydration.

- Remember that influenza and bacteria can and often do co-infect.
  - Really sick patients may have a bacterial superinfection facilitated by the virus.
(Potential) Benefits of Flu Testing

**For positives...**
- Rapid treatment.
- Avoidance of antibiotics and costs and complications thereof.
  - We all know what a large fraction of antibiotics are used for viral infections.
- Avoidance of further workup / admission in some cases.
  - How much will test impact this versus clinical condition of the patient?
- Infection control – inpatient and outpatient.
- Patient flow in outpatient settings:

  All these depend on a result provided within the encounter time or shortly thereafter.

**For negatives...**
- Save cost of antiviral therapy.
- Save isolation cost / inconvenience
- Continue diagnostic workup if patient’s condition warrants it.
Influenza Specimen Collection

Specimen collection is probably the critical step in influenza testing.

- **Good test on a bad specimen = bad test**

Nasopharyngeal Wash: Syringe Method

**Materials:**
- Saline
- 3-5 ml syringe*
- 2” 18-20 gauge tubing*
- Viral Transport Medium (VTM)
- Specimen container

1. Fill syringe with saline; attach tubing to syringe tip.
2. Quickly instill saline into nostril.
3a. Aspirate the recoverable nasal specimen. Recovery must occur immediately, as the instilled fluid will rapidly drain.
3b. *(Alternate)* In appropriate cases, patients may tilt head forward to allow specimen to drain into suitable sterile container.
4. *(If aspirated)* Inject aspirated specimen from syringe into suitable dry, sterile specimen container or one containing VTM, according to virology laboratory requirements.

*Length and diameter of bulb as appropriate for infant, child or adult.

Washes are somewhat better than swabs*

*A general but not-quite universal rule of microbiology: swabs are evil*
Specimen Collection – The NP Swab

- NOT A THROAT SWAB. NOT A NASAL SWAB. A NASOPHARYNGEAL SWAB.

- Important to get ciliated epithelial cells – this is a cell-associated virus

- Test early; more virus is shed early than later in disease.

- A test a week after onset of symptoms is useless.

- Children shed more virus than adults

- Tests tend to be more sensitive in kids

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**Nasopharyngeal Swab Method**

*Materials:* BD BBL CultureSwab flexible, soft, or regular aluminum wire products *or* Nasopharyngeal swab with synthetic fiber tip 1-2 ml Viral Transport Medium (VTM) Specimen container

1. Insert swab into one nostril.
2. Rotate swab over surface of posterior nasopharynx.
3. Withdraw swab from collection site; insert into transport tube or container with VTM.
Managing POC Molecular

- All the usual QC and QA, plus:
  - Interferences
    - Extraction efficiency
    - **Inhibition** by:
      - Blood
      - DNA
    - Internal amplification / extraction controls
  - Contamination
    - Extraordinarily sensitive methods
    - **Specimen** cross-contamination
      - Native material transferred from a positive to a negative specimen
      - Collection devices
      - Ports, racks, hands
    - **Amplicon** contamination
      - From amplified material
      - How well is the product contained?
      - Waste disposal
    - Carry-over studies
Future Developments

- Technological advances
  - performance
  - speed
  - footprint
- Expanded test menus
  - quantitative assays
- Resource limited settings
Where are we going?

- I’ve thought about this a lot.
- Derived Campbell’s Laws of POCT
- Two Laws, with inpatient and outpatient corollaries
  - Feedback encouraged.
Campbell’s First Law of POCT

Nobody ever went into Nursing because they wanted to do lab tests.

I can’t document this with a literature citation, but it has high face-validity.

Anecdotally, our nurses/docs have hated glucose monitoring (still done but loathed), ER troponins (tried, failed), and rapid HIV (tried, failed).
Campbell’s Second Law of POCT

- No POC test is easier than checking one more box on the laboratory order form.
  - Waived tests are easy, but much, much harder than checking one more box on a form you already filled out.
  - A lot of simple, rapid tests end up being done in the lab.
Campbell’s Laws Example: Primary Care HIV Testing

- **June 8, 2010:** Provider A: “Sheldon, has rapid testing been considered to prevent this problem? Would this be feasible? Might allow us to expand testing to highest yield sites (i.e. the ER)...”

- **July-October 2010:** Set up program, created templated progress notes, ordered kits, trained 20+ Primary Care providers to do rapid HIV tests.

- **October 2010-January 2011:** Number of rapid HIV tests performed: 1

- **January 2011:** Provider B: “Even though I am one of the biggest proponents, I have only done one, and that was for another provider who didn’t know how to do it. I don’t see people clamoring to do the test. I’m interested in Provider A’s thoughts.”

- **Response, Provider A:** “We have had very little use in <our clinic>. I think that it’s so easy to send the pt for bloodwork that there is not much demand.”

- **January 7, 2011, POCC:** “Next week I will be coming around to the Primary Care areas to collect the HIV kits. Please have them easily accessible. Thank you and have a pleasant weekend.”
Campbell’s Laws: Inpatient Corollaries

- An inpatient POC test is useful only if:
  - The time for transport to the lab for THAT SINGLE ANALYTE significantly and negatively impacts care, OR
  - The test is performed on an easily-obtained sample (e.g. fingerstick blood) more frequently than routine blood draws are obtained.
Campbell’s Laws: Outpatient Corollaries

- An outpatient POC test is useful only if:
  - The test result is available during the patient visit AND a decision can be made or action taken on the basis of it without waiting for other lab results, OR
  - If you can make money doing it.
Campbell’s Outreach / Developing-World Corollaries

- Sometimes there’s no lab-order form.
- Sometimes there’s no nurse.
- Sometimes there’s no refrigeration, power, or lights.
- Campbell’s Laws should not be applied outside of a healthcare environment where the basic terms apply.
Recommendation

“Point-of-care testing, especially those analyses that are conducted at the patient’s bedside, in a physician’s office, or in a clinic, is a growing trend in health care, and clinical microbiology professionals should prepare for this future reality. Clinical microbiologists must ensure that the individuals who perform point-of-care testing understand how to interpret the results. Clinical microbiologists should be called upon to help select the assay targets, advise on test formats, and participate in clinical trials.”