The Good, the Bad and the Ugly! Pitfalls

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Movement Toward Molecular Microbiology

• Current times still rely on traditional culture based methods for diagnoses
  • Culture based technologies needed due to variety and complexity of specimen types
  • Demand for a large variety of pathogens
  • Require multiple media types, incubation temperatures and stains

• Progressive movement from traditional microbiology to molecular methods

• Development of successful technologies, that
• Do remarkable things and have brought much good to patient care when applied appropriately
• But with The Good, has also brought a bit of the Bad and the Ugly!
• The story continues.......
The story will include:

- Appropriate specimens for analysis
- Performance characteristics of different molecular methods
- Optimal specimen processing
- False positive results from contaminating nucleic acids
- Effects of PCR inhibitors
- Too much sensitivity in some cases
- Problems with monitoring therapy
- Cost effective test use
I. Appropriate specimens for analysis

Example: GC/CT molecular analysis using Cobas® 4800 system

The Good: FDA cleared specimen types acceptable for use in well established assay with high sensitivity/specificity for GC/CT detection

- **Urine** (male/female) acceptable for up to 24 hours in sterile cup or in cobas® urine media added to fill line

- **Cervical/vaginal** – collected with cobas® female swab kit, self or physician collected

- **Pharyngeal** and anal/rectal specimens collected with either cobas® collection kit or Universal transport media (UTM) self or physician collected

- **Endo cervical** specimens collected in SurePath™ liquid medium
Tales of woe, the Bad

• Friday late afternoon patient, suspicious for GC/CT the hour is late, urine specimen is collected and refrigerated for send out on Monday, arrived at reference lab >48 hours after collection
  • This falls outside the allowable FDA specimen requirement, urine specimens must be tested or aliquoted into cobas® transport medium within of 24hrs of collection / The urine should be rejected for analysis

• Urine is collected and then added to cobas® urine medium tube, the volume is not between the designated fill lines
  • The correct amount of urine was not added to the tube /The urine should be rejected for analysis

• Immediate notification of rejection / but patient is gone!
Tales of pitfalls – self collection

• Assessing adequacy of self collected vaginal and rectal swabs
  • Are the collection instructions followed?
  • No way to assess the quality of self collected specimens in the laboratory for testing
• Problem specimens received from self collection
  • Feces on a rectal swab collected for GC/CT
  • The clumps clog pipettes in the instrument/ no result
  • Specimen must be rejected
Why not run the assay anyway?

- It is only a day or two longer, what’s the deal with fill lines or feces
  - Would be “off label” with analysis, not using a specimen within the published regulatory guidelines for established test performance
  - Place the laboratory at risk
  - No evidence that the result would be reliable

- **The Bad /** more than one test method for a designated pathogen
  - Each company has different & specific collection requirements
  - Solution: Specimen collection guidelines and collection devices need to be immediately available for test method for the destination laboratory
Why not Modify the Specimen Type?

• For example: Multiplex PCR Respiratory Viral Panel
  • FDA cleared specimen type: Nasopharyngeal swab in Viral transport media

• Bronchial Lavage (BL) is a reasonable diagnostic specimen for a serious respiratory infection, but not accepted by FDA as an acceptable specimen type – the Bad

• To offer BL specimen, requires validation study for modified specimen
  • Literature review / does this specimen type work in this assay?
  • Cost: Labor, “validation” panels and/or acquire clinical specimens
  • Perform validation testing and documentation
  • Level of expertise needed for validation design and performance to pass regulatory demands
II. Performance characteristic of different molecular methods

- FDA cleared test
  - Defined multi-center evaluation approved by FDA and findings published for review
  - To use in a laboratory you must perform in house verification (>=20-30 specimens), document CLS training, and competency, ongoing QC
  - Most/all assays being tested in community microbiology laboratories

- Laboratory developed test (LDT) –
  - FDA cleared individual analyte specific reagents (ASRs) are used but a single laboratory develops test / performed in that one laboratory / medically/legally responsible for test performance
  - Why do this? FDA cleared test is not available / Less expensive / Rare emerging pathogen
  - How rigorous of an evaluation can be done on a rare or emerging pathogen?
  - **The Good / The Bad / ?big problem** - FDA continues to lead discussions for the need to monitor the quality development of LDTs
Performance Characteristics of Different Molecular Methods – or do these tests work?

Problem: Lack of a suitable gold standard for comparing assays

• Premise: Molecular test is more sensitive than culture
  • Stool specimen: Molecular test is POSITIVE for Shigella /Culture is NEGATIVE
  • Increased sensitivity of molecular test or is this a false positive reaction?

• So ideally you need a true “comparative assay” to define performance:
  • What are “good” specimens for test comparison/ frozen specimens OK?
  • Each FDA clinical trial or study may validate the performance of an assay differently, so how do you compare their performance
  • Is a previously validated molecular assay that has a different molecular target a good comparison to judge the quality of a new molecular assay?
  • Should everything be compared to 16s RNA sequencing
  • There may be very few clinical studies published to review
Performance of assays – What does it mean?

• Rapid detection of Mycobacterium TB complex organisms from sputum specimens by PCR / also detects rifampin resistance gene (rpoB)
  • MTB complex – not specific for TB, detects M. bovis (part of complex)

• Sensitivity for detection of MTB complex¹
  • Sputum with positive AFB smear  98% sensitivity in PCR assay
  • Sputum with negative AFB smear  72% sensitivity in PCR assay
    • Superior to microscopy for rapid detection of MTBC by 23%¹
    • However, does eliminate the possibility or MTB complex in sputum specimen

• Test of diagnosis/ not test of cure, detects nonviable organisms/ patient on therapy will remain positive (for up to 6 months)
Performance of assays – do all assays the perform the same?

- Four FDA approved/cleared multiplex respiratory PCR panels – compared using 300 specimens
- Comparable specificities (99-100%)
- Variable sensitivities for overall detection of viruses on these panels from low 84.5% (1) to high 98.3% (2)
- Greatest number of discrepancies between the multiplex panels and the reference standard was for Influenza B and Adenovirus
- Due to inferior performance on CAP proficiency testing samples, the FilmArray panel was redeveloped for Adenovirus which included more viral serotypes

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<thead>
<tr>
<th>Study</th>
<th>Popowitch et al (1)</th>
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<tr>
<td></td>
<td>Number of positives (n=300 samples tested)</td>
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<td>Adenovirus</td>
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<td>Respiratory syncytial virus B</td>
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<tr>
<td>Rhinovirus/enterovirus</td>
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The company recognized that improvement was necessary and added additional Adenovirus serotype targets onto the panel. The improved sensitivity was improved to <=85%.

- Just because a test is approved by the FDA / do not assume that the sensitivity of targets are approaching 100%
- Prior to adopting a test in the laboratory, or relying on the panel result as the only diagnostic test, need to review the FDA clinical trial data and published studies to access possible shortcomings in the assay.
Performance of assays - Multiple organisms detected / what organism?

- Multiplex GI panel detects of $\geq 2$ targets occur far more often than with conventional testing ($\geq 50\%$ of the time)
- Samples with multiple pathogens
  - $84\%$ positive for Enteroaggregative Escherichia coli (EAEC) and Enteropathogenic E. coli (EPEC)
- Are these pathogens?
- Current guidelines provide no direction for treatment or clinical significance, or need for confirmatory testing
- Studies have also shown unexpected co-infection that includes C. difficile and Norovirus
- Rare pathogens / how were studies performed?
- Adoption of this assay will come with questions – Ready?
We are using an awesome new multiplex PCR for enteric infections.

It detects a ton of pathogens!

Wow! You report all of them?

Well, not *Clostridium difficile*. We have a separate test for that.

And not those that are common in the healthy microbiota.

Uh-huh.

And not *Vibrio* species... too rare for decent positive predictive value.

Party pooper.

Uh-huh.

Performance of assay – did we contaminate this specimen?

Sensitive assay using PCR for the detection of the most common bacteria, viruses and yeast causing meningitis and encephalitis from a CSF specimen

One unusual finding in clinical trial data: 7/16 *Streptococcus pneumoniae* were proven to be discordant false positive reactions
Relative performance of the FilmArray ME Panel versus comparator assays after additional discrepancy investigation.

Strep pneumoniae detection with the most discrepant results

Not the only discrepant problem detected
Investigation into Discrepant Results

- **Investigation**: Difficult to determine exactly how it happened, but the testing site with most issues did not use a biological safety cabinet and was not experienced in molecular testing or sterility practices.

- **Determined** to most likely be contamination of the specimen during aliquoting and testing at the clinical trial site:
  - Possible carryover from processing in all areas of the laboratory and/or contamination from normal flora of the study personnel.

- **Program** to monitor sterility practices and review all molecular test results for contamination trends and quickly resolve problems.

- So this leads into.......
III. Optimal specimen processing – or how is that specimen being handled in the lab

• Marvelous, moderate complexity multiplex and single target molecular assays available for microbiology laboratories
• Marketed that performance is quite simple and not time demanding
• The Bad: Placing molecular testing in the hands of skilled plate readers that have never been fully trained/ nor have experience in molecular testing
• The skill sets for molecular testing and manual microbiology are different
  • Molecular sterility practices may appear tedious to manual microbiologist
  • Bleach and 70% alcohol need to become your best friends
  • Work performed in “clean” biosafety cabinet / for protection of worker, but also protection of the sterility of the specimen
  • Pipetting never pouring
  • Proper use and changing of gloves between steps .................
High complexity vs Moderate complexity testing

• **High complexity** – so called because they are HARD to do!
  • These assays were the first to reach the market @ 15 years ago
  • Off line extraction, open processing, pipetting tiny volumes, multiple instruments to care for with concern for contamination
  • Best in the hands of molecular skilled, not microbiologists whose primary training includes reading agar plates and performing Gram stains

• **Moderate complexity**
  • Sample to result, automated assay
  • Easier to perform steps and microbiologists do not struggle with performance
  • Introduced as assays that anyone could perform, some even point of care
Is the moderate complexity assay “simple”?

- Manufacturers learned that laboratory training on a new “simple assay” demanded both teaching the performance of the assay plus education on sterility practices.

- Simple in performance and easy to contaminate.

- Even the most simple of assays could be contaminated by sloppy technique or a “contaminated” biosafety cabinet.

- These are amplification assays that detect miniscule amounts of genetic material.
Additional problems and challenges for the microbiology laboratory

• Educating staff to learn new skill while maintaining skills in classic microbiology – multi-tasking

• **Answering physician questions** – stretched in many directions, may not be able to quote the package insert or newest literature

• Interpreting test results can be challenging for the CLS
  • They may be quite skilled in the performance of the test
  • Not as skilled in the difference between Shigella, EHEC, and Shiga toxin

• So for problems who you going to call?
  • Hopefully, there is a laboratorian skilled in this area awaiting your phone call
IV. False positive results from contaminating nucleic acids outside the laboratory

Aerosolized vaccine as an unexpected source of false positive B. pertussis nasopharyngeal specimen PCR results in children

- BP vaccine has high copy #’s of DNA / aerosolized during preparation and administration
  - Health care workers preparing and administering vaccine in the same room where patient specimens were collected for possible pertussis infection¹
  - Specimen contamination at the point of specimen collection, environmental contamination, poor specimen collection practices/ poor environmental cleaning, use of liquid aerosolized transport media, LDT without relevant cutoff values²

1. H. Salimnia, 2012 JCM, 50(2)  
2. S. Mandal, 2012, Pediatrics, 2012, Feb 129(2)
V. Effects of PCR Inhibitors

- **PCR inhibition** is the most common cause of amplification (test) failure when sufficient copies of DNA are present, substances interfere with different steps of the PCR analysis.
- Usually affects low % of clinical specimens.
- Vagisil® and gel lubricants used in specimen collection are common inhibitors.
- Inhibitor = amplification failure = no result.
- Perhaps another molecular test may not have the same inhibitory substances that cause problems – inhibition can be somewhat method dependent but usually better specimen collection is needed.
Every test has list of interfering substances in the FDA package insert

Interfering Substances

The presence of PCR inhibitors may cause false negative results.

Interfering substances include, but are not limited to the following:

Replens® Lubricant has been shown to inhibit PCR and may yield false negative results with this Test.

The presence of mucus in cervical samples may inhibit PCR and cause false negative test results. Mucus free samples are recommended for optimal test performance. Use a sponge or a large swab to remove cervical secretions and discharge before obtaining the sample.

Samples containing greater than 5% (v/v) blood may give false positive results. In Clinical studies, 681 of 2265 female swab specimens were noted to be bloody. False positive rates were not higher in these specimens.
VI. Too much sensitivity – could this be colonization and not infection?

• Detecting *C. difficile* in stool specimens using PCR
  - PCR detects tcdB gene in stool / present with or without active toxin production
  - So……Potential problem separating disease versus colonization?
  - PCR alone *may* be adequate for detecting disease, if you can control the near perfect selection of patients with true diarrhea¹
  - If patient/stool selection is somewhat suspect (*normal situation*) current IDSA Practice Guidelines suggest using a 2 step reflex algorithm¹
    - Step 1: PCR assay to detect tcdB gene
    - Step 2: PCR positive specimen, reflexed and tested using EIA for toxin A/B

¹Clinical Practice Guidelines Clostridium difficile 2017
Diagnostic Algorithm for Detection of Toxigenic C. difficile in Stool using NAAT

Perform BD MAX Cdiff panel

Not Detected

Colonization
Physician has information for treatment decision

Detected

Reflex to C diff toxin A/B EIA

Infection
Patient requires treatment

PCR+/EIA-

PCR+/EIA+
VII. Problems with monitoring therapy if assay detects dead organisms

• Diagnostic test, unable to monitor therapy or use as test of cure

• No way for laboratorian to determine if the organism detected in a molecular assay is dead or alive
  • Remnant DNA and dead organisms are as easily detected as live ones
  • FDA cleared assays in use are qualitative not quantitative

• Clearing DNA and converting back to a negative PCR result:
  • Differs for each pathogen and individual patient
  • For example: TB/RIF PCR assay can remain positive for up to 6 months following the start of therapy
VIII. Cost effective test use – or who is paying for all of this?

- Target Utilization
  - Pathogen or disease targeted algorithms to assist with molecular assay utilization such an algorithm was created for stool testing at Mayo Clinic¹

- To support such an algorithm need data support:
  - Is the use of broad multi-plex panels most helpful to the patient and worth the excessive cost
  - Is a more limited approach using a targeted algorithm with less testing both benefit the patient and healthcare cost

¹ P. Ramanan, Syndromic Panel-Based Testing in Clinical Microbiology, JCM (31)1 Jan 2018
What about the cost of this technology – is it worth the increased cost?

- **Cost to the laboratory $$$ - $$$$$**
  - Instrument / preventative maintenance contracts
  - Reagents
  - QC processes
  - Staffing – training and competency

- **Offset by the question – is there a benefit to the patient?**
  - Increased costs in the laboratory must be offset by improved patient care
  - Needs to be data driven and convincing or the laboratory cannot obtain or keep $$$ testing
  - The laboratory usually loses in these power games/ the only hope is to form alliances with Stewardship/Infectious Disease
Who decides if we are paid or NOT paid?

Noridian denies coverage effective May 13, 2019
Manufacturer granted PLA codes for multiplex panel billing/ reimbursement approximately 40% of previous amount using prior CPT coding

Reimbursement for large multiplex panels does not look favorable over time
In summary

• Movement toward molecular microbiology

• Remarkable technologic advancements in automated molecular methods that are advertised to be easy enough for anyone to perform

• Cautionary tale
  • The Good – Provide rapid detection of pathogens to improve patient care
  • The Bad – Not all tests are created equal
    Need to be aware of some possible pitfalls in select tests
    Quality specimens are essential for test sensitivity
  • The Ugly/Pitfalls – Even the most simple of tests can be poorly performed
    Laboratorians must perform tests with care using proper sterility practices
    Cost effective test use is essential, reimbursement is a moving target!