Today's Featured Speaker

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Polling Questions

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
  - Distinguish between real-time and non-real-time molecular methods
  - Recognize the properties of an analyte that make it a candidate for molecular testing e.g. discrete, single or small # of targets, present in samples…do an easier-harder kind of organization.
  - Recognize emerging molecular diagnostic platforms that may be usable at point-of-care
  - Describe unique quality issues in molecular diagnostics which impact their use at point of care e.g. contamination, inhibition, sampling

Outline

- What is molecular diagnostics, exactly?
- Why would we want to do POC molecular testing?
- Molecular diagnostics technologies.
- What analytes make sense?
- What platforms are out there?
- What unique quality issues will there be?
- Thinking about the future.

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

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

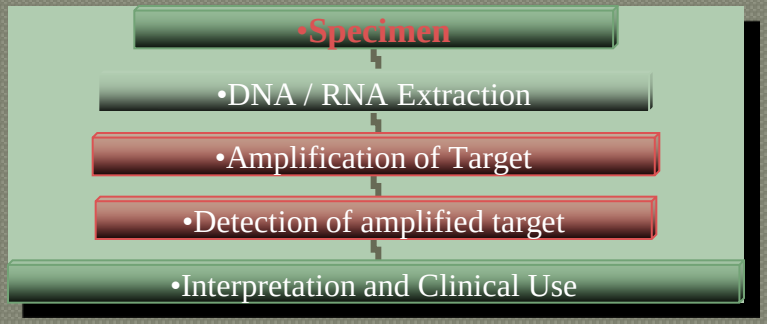
- Targets
  - Test for things there's no other way to test
  - Uncultivable bugs
  - Genetics
    - Pharmacogenomics
    - Prenatal testing
    - Hypercoagulability, etc.
  - Oncology
    - Hematologic malignancies
      - Diagnostic markers
      - Minimal residual disease

Why Not Amplify?

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

Specimen Sources

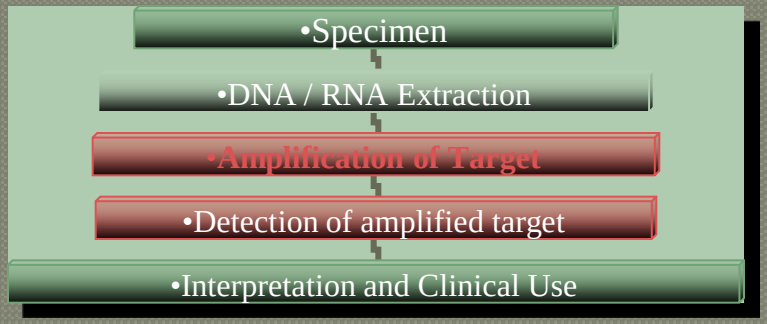
- Blood/Serum
  - heme and hemelike compounds strongly inhibit
  - pathogens in low concentrations
  - anticoagulants (heparin, EDTA, citrate) inhibit
  - serum proteases can be inactivated by heating
- Urine
  - amorphous salts during storage make purification difficult
  - urinary inhibitors vary widely
- CSF
  - spun pellets often contain high inhibitor concentrations
- Sputum
  - can contain huge amounts of DNA (up to 14 mg/ml)
- Stool
  - the most difficult specimen
  - many inhibitors, large background of bacterial and cellular DNA



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

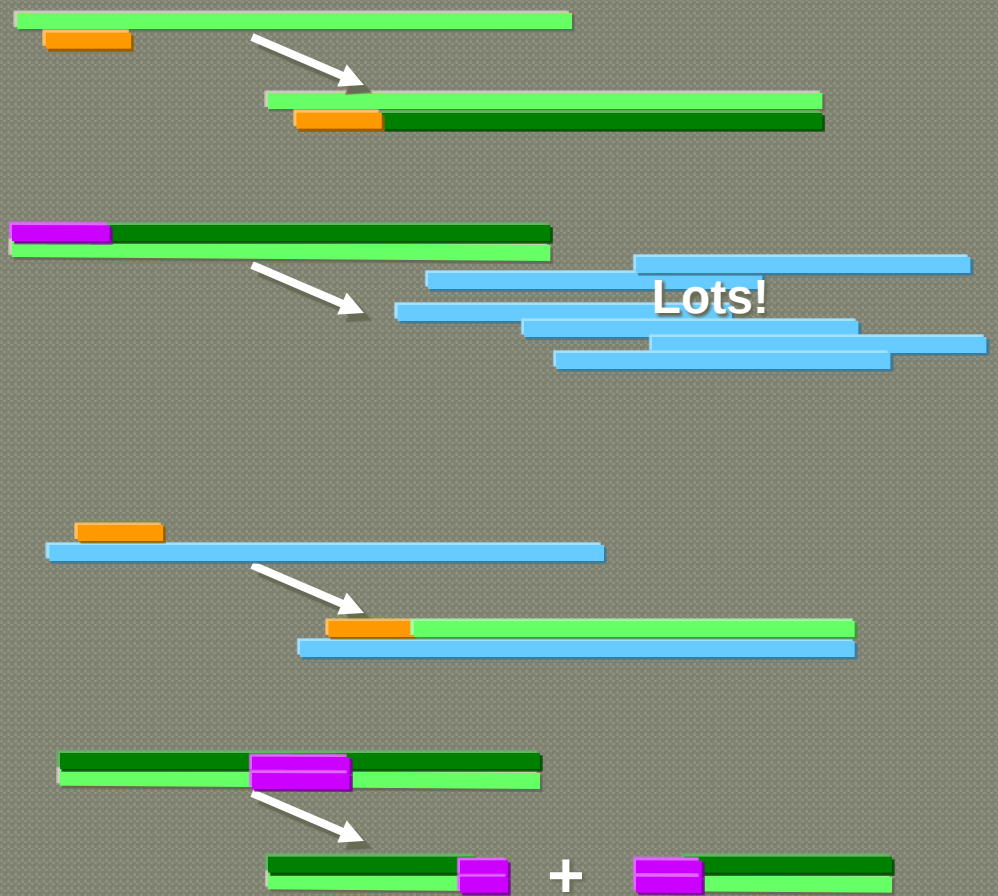
Amplification



- 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



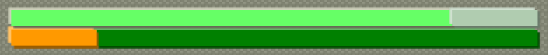
Polymerase Chain Reaction (PCR)

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



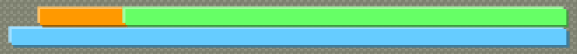
This ‘short product’ amplifies exponentially in subsequent split-bind-extend cycles, driven by the temperature changes in a ‘thermal cycler’.

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

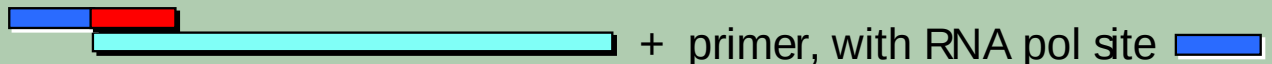


The DNA copy is used in a PCR reaction

PCR

Transcription-Mediated Amplification

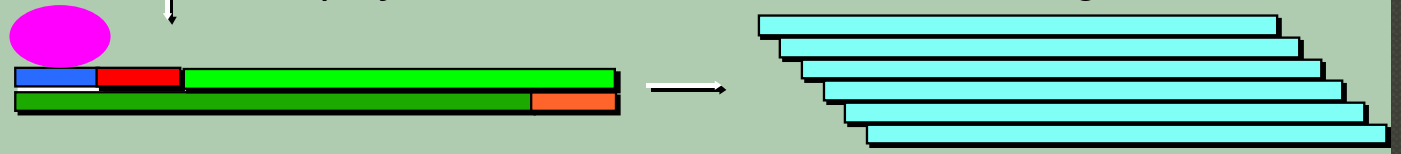
Target= RNA or single-stranded DNA



reverse transcriptase makes DNA from the RNA

split strands (95°C 5 min), then anneal second primer, which is extended by the reverse transcriptase

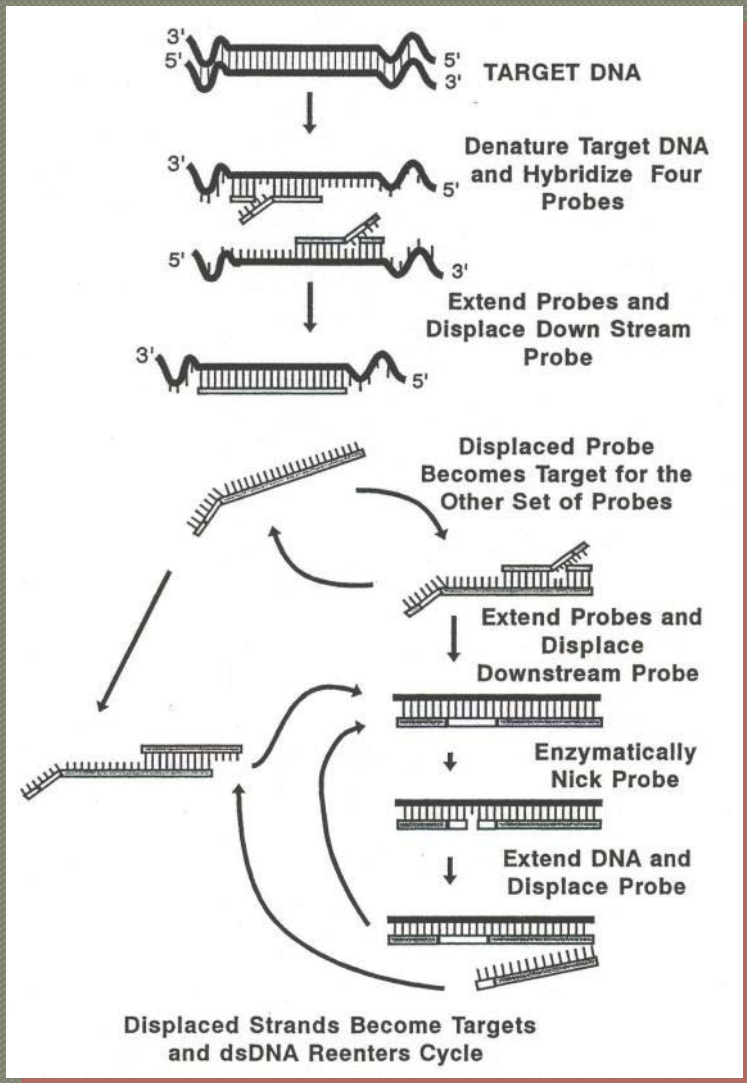
RNA polymerase transcribes 10-1,000 new target RNAs



A small number of cycles can produce a 10^6 fold amplification

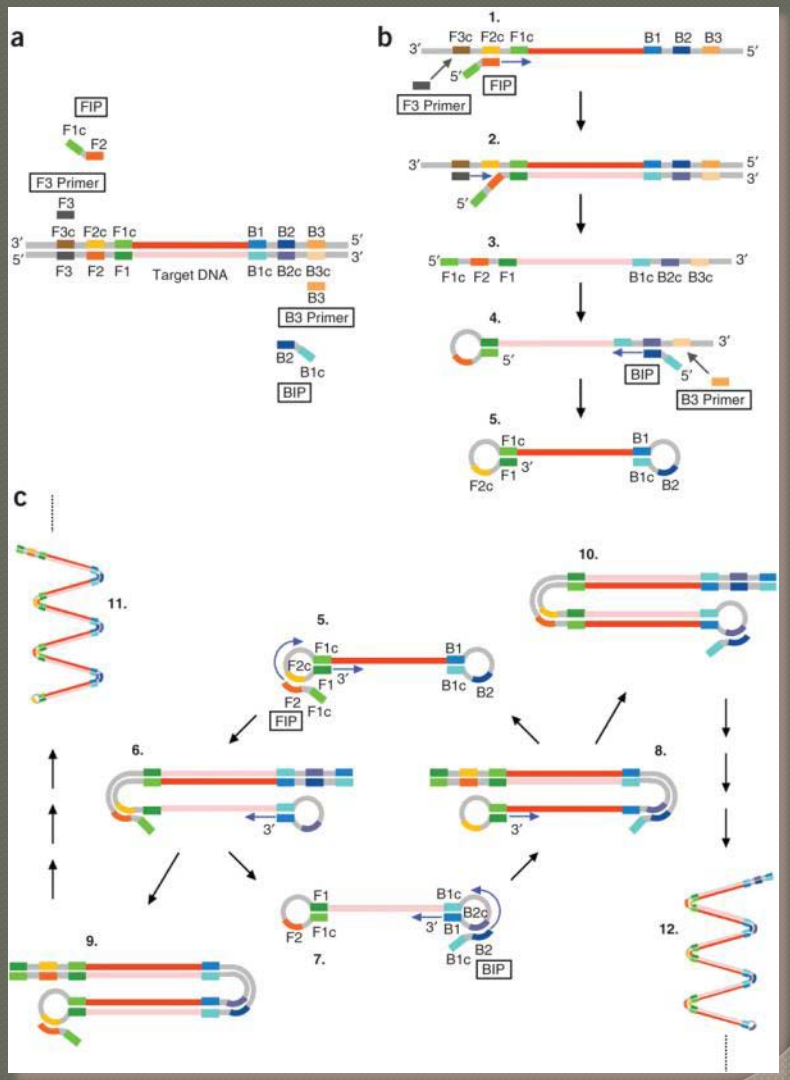
Strand-displacement Amplification

- Complex
- But it works

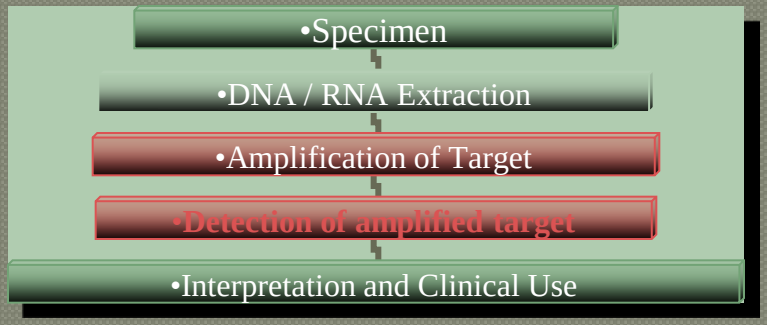


LAMP

- Loop-mediated isothermal AMPlification – LAMP
- Makes long products which can be easily detected by turbidity or fluorescence.
- Requires no thermal cycling
- Well-adapted to POC use.



Detecting PCR Products



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

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.
- Almost always use PCR for amplification
  - Robust
  - Off-patent

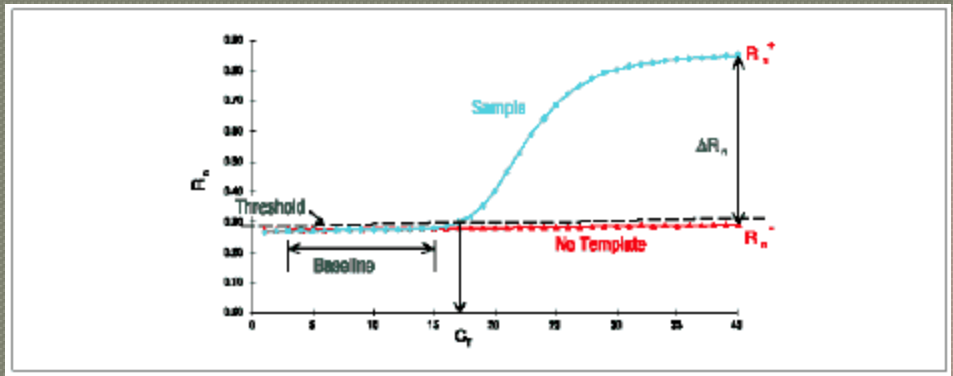


Figure 2. Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR Figure from Applied Biosystems' DNA/RNA Real-Time Quantitative PCR bulletin).

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.

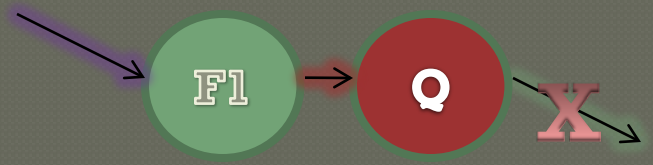
Real-time PCR Chemistries

- 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

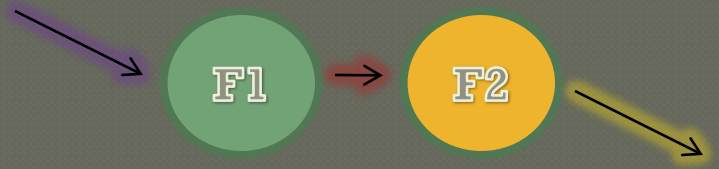
- Quenching
  - Fluorescence occurs when a photon bumps an electron to a higher energy level, then another photon is emitted when it drops back to ground state.
  - Some compounds ('quenchers') suck up that energy before it can be reemitted, 'quenching' the fluorescence.



  - This is distance dependant; the closer the molecules are the more efficient the quenching.

Fluorescence Resonance Energy Transfer (FRET)

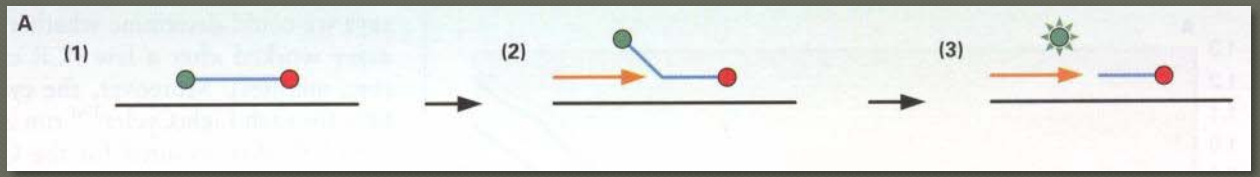
- A second fluorochrome can suck up the energy from the activated fluorochrome and re-emit it at its emission frequency.



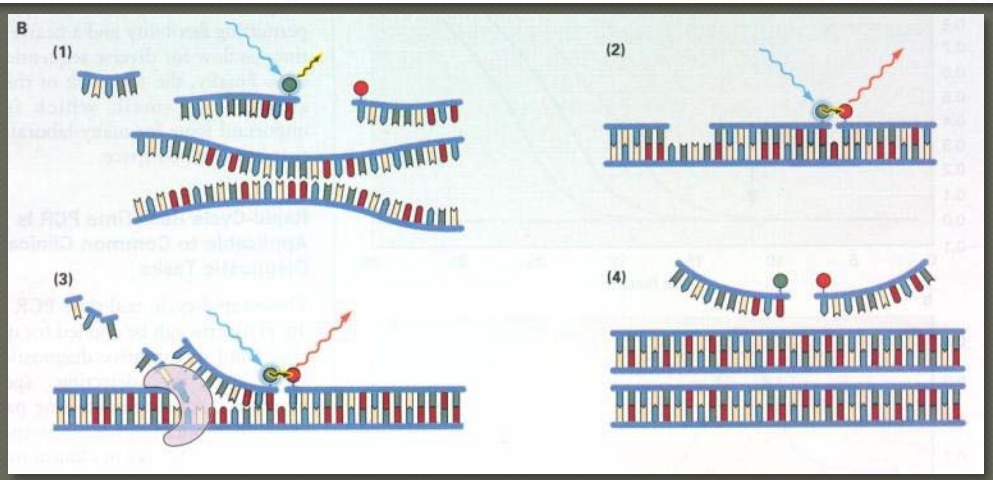
- This is distance dependant; the closer the molecules are the more efficient the energy transfer.

Real-Time Detection Schemes

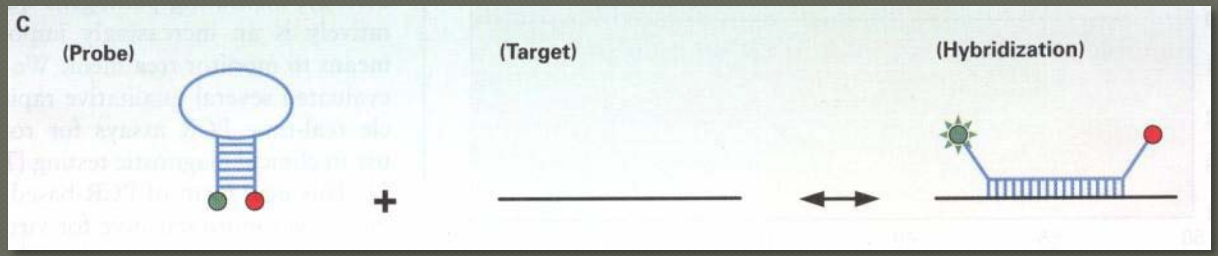
- Taqman Probes



- 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 μm 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*
  - UNG

POC Molecular Diagnostics

- Infectious Disease
  - Outpatient POC
    - GC / *Chlamydia*
    - Group A strep
    - HIV / HCV viral load
  - 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 form (mostly pre-approval). The rest are guesses.

Thinking about the Targets

- Single targets are easier than multiples
  - Even single targets may require multiple primers and probes due to polymorphisms
    - One MRSA test uses 7 primers and 5 probes!
- Genetic targets are easier than microbes
  - Easier to get large amounts
  - Easier extractions
- Qualitative tests are easier than quantitative
  - *Chlamydia* vs. HIV viral load; bcr-abl for diagnosis vs for disease monitoring.

Why Molecular? Rapid flu versus Other Methods

Influenza A Rapid Test Performance¶

Rapid Test¤	Sens%¤	Spec%¤	Compared With¤	Comments¤	Reference¤
Directigen ¤	58.8¤	99.2¤	Molecular¤	A&B performance combined¤	Liao et al JCM 47(3):527-32, 2009 Mar¤
3M↵ QuickVue ↵ BinaxNow¤	75↵ 73↵ 55¤	98↵ 99.5↵ 100¤	Culture¤	Archived specimens¤	Dale et al JCM 46(11):3804-7, 2008 Nov¤
BinaxNow¤	53¤	¤	RT-PCR¤	2 of 237 samples were flu B pos by RT-PCr but flu A by NOW. ¤	Landry et al JCV. 43(2):148-51, 2008 Oct¤
BinaxNow¤	61¤	100¤	RT-PCR¤	DFA was 81% sensitive¤	Rahman et al Diag Micro Infect Dis 62(2):162-6, 2008 Oct¤
RemelXpect↵ BinaxNow¤	47.7↵ 78.3¤	98.7↵ 98¤	Culture¤	20.3/99.8 Flu B↵ 35.9/99.9 Flu B¤	Cruz et al JCV 41(2):143-7, 2008 Feb¤
BinaxNow¤	52¤	¤	RT-PCR¤	70% in days 1-3 of disease¤	Nilsson et al Inf Cont & Hosp Epi 29(2):177-9, 2008 Feb¤
Directigen ¤	42¤	96¤	Culture¤	¤	Rahman et al Diag Micro Infect Dis 58(4):413-8, 2007 Aug¤
BinaxNow↵ Directigen↵ QuickVue¤	73↵ 69↵ 67¤	99↵ 100↵ 100¤	RT-PCr¤	Sensitivity only 30% vs flu B for all¤	Hurt et al JCV 39(2):132-5, 2007 Jun¤
Quickvue¤	85¤	97¤	RT-PCR¤	¤	Mehlmann et al JCM 45(4):1234-7, 2007 Apr.¤
Directigen + Quickvue + BinaxNOW¤	63¤	97¤	RT-PCR¤	Data pooled from all rapids; ¤	Grijvala et al Pediatrics. 119(1):e6-11, 2007 Jan¤

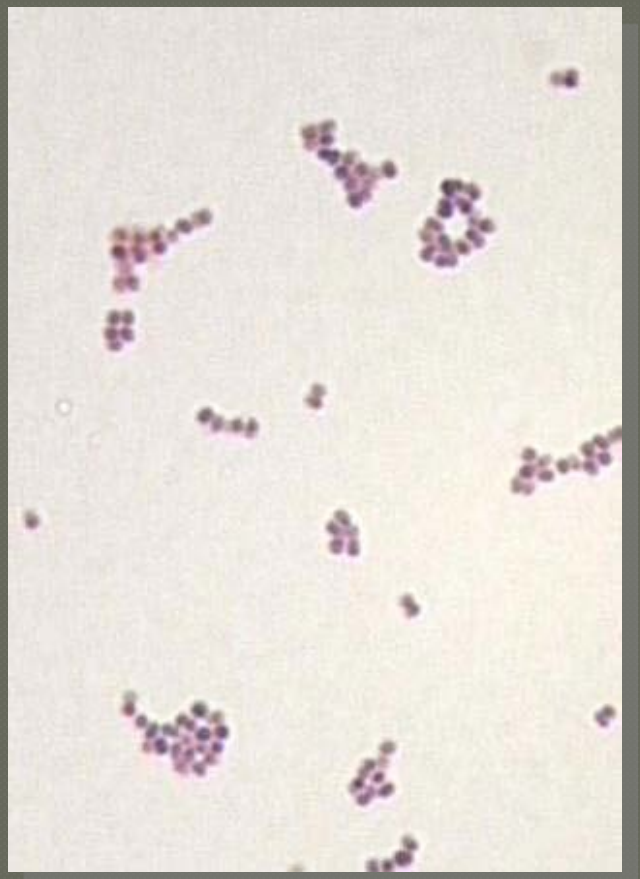
Convenience sample of recent literature; selected by Medline search + fit to single page

What Will Be First?

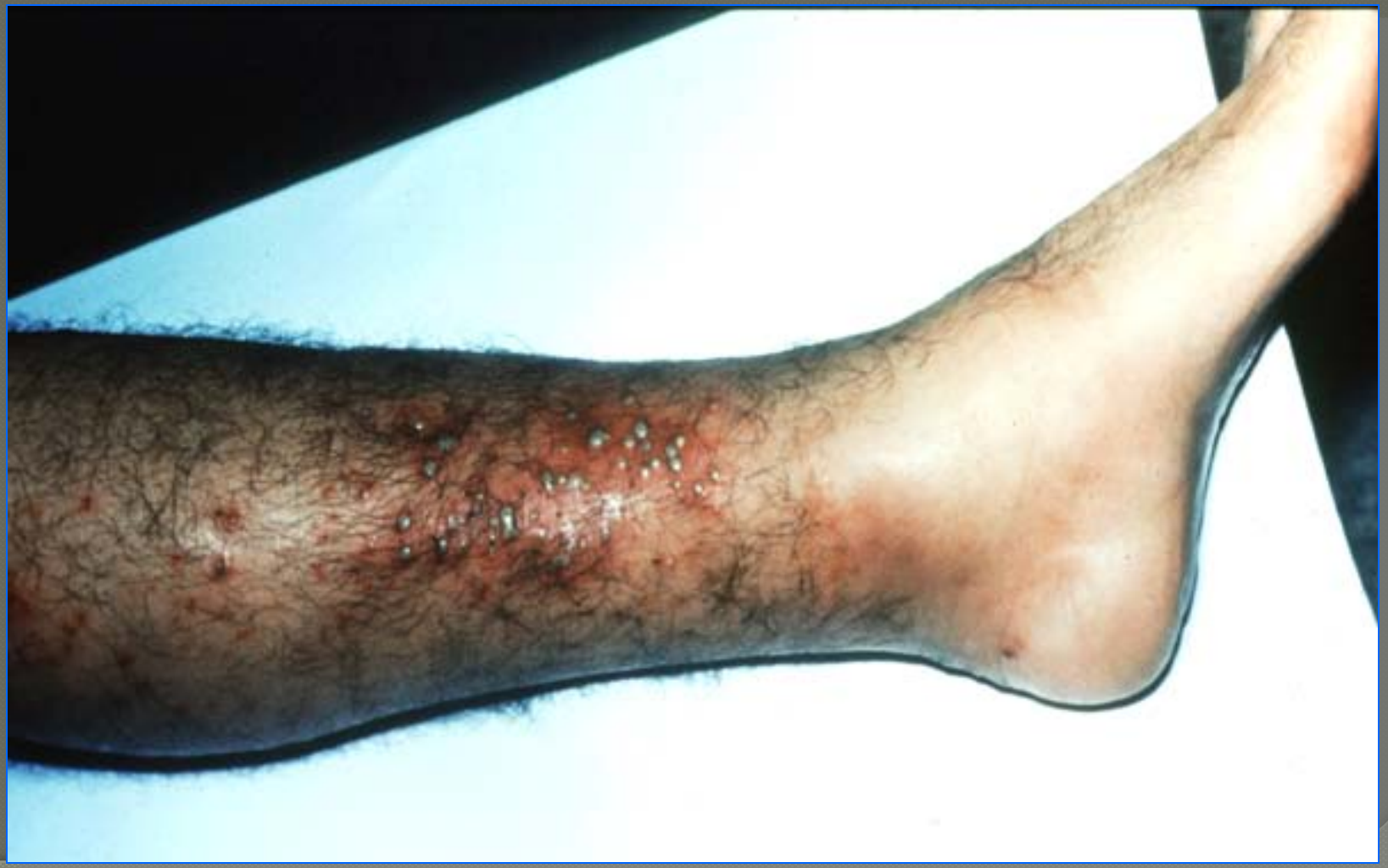
- Things that're easy
  - MRSA, already on GeneExpert
- Things that're hot
  - Influenza
- Things where existing tests perform poorly
  - Respiratory viruses in general
  - Group A strep
  - Group B strep

Staphylococcus aureus

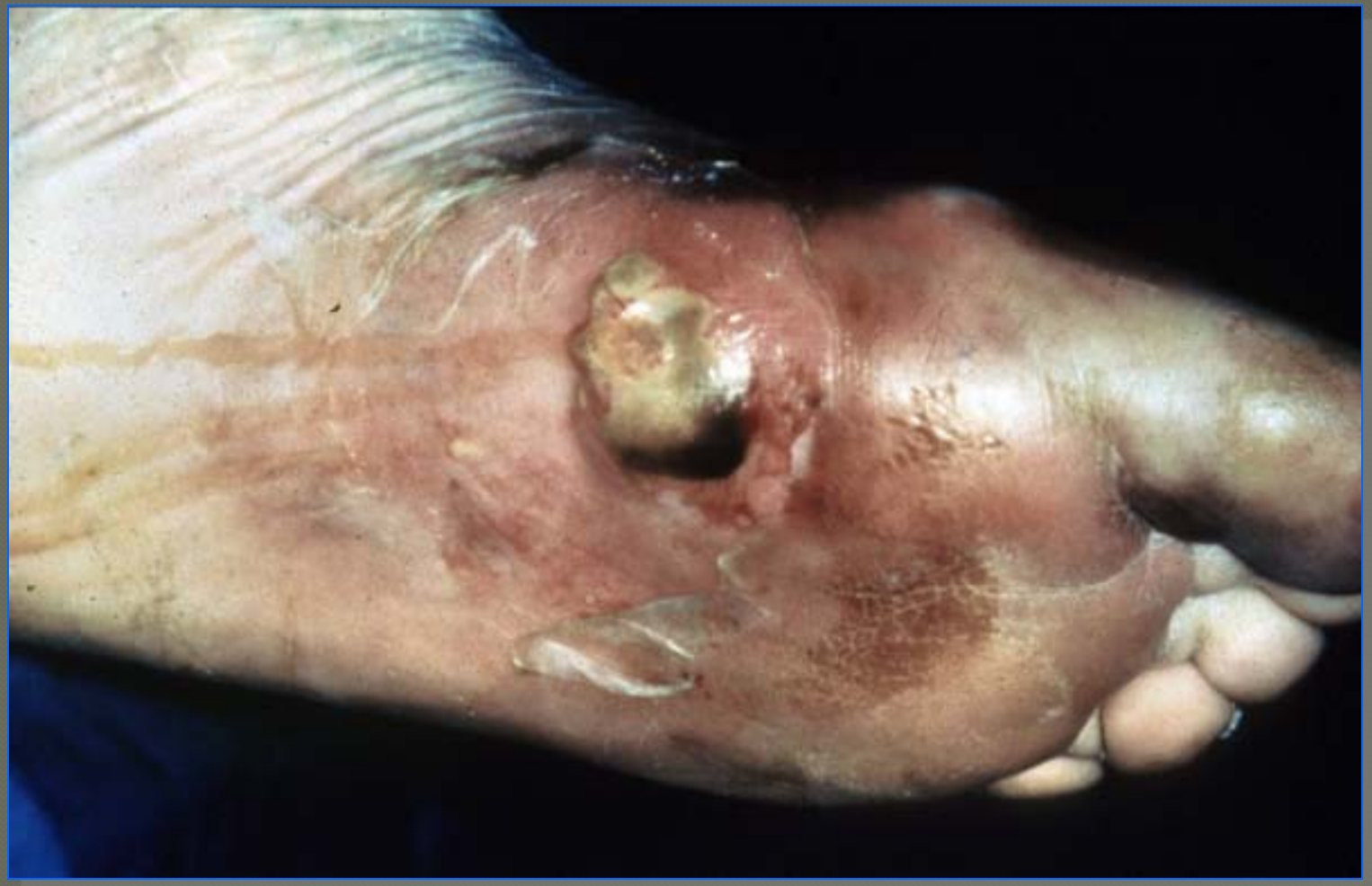
- Major nosocomial and community-acquired pathogen
  - Responsible for >20% of bacteremia in US/Canada
  - Transmissible nosocomially and in the community.
- Gram-positive cocci in clusters
- Sepsis, pneumonia, skin, wound, and soft tissue infections, osteomyelitis, UTI, endocarditis, etc.



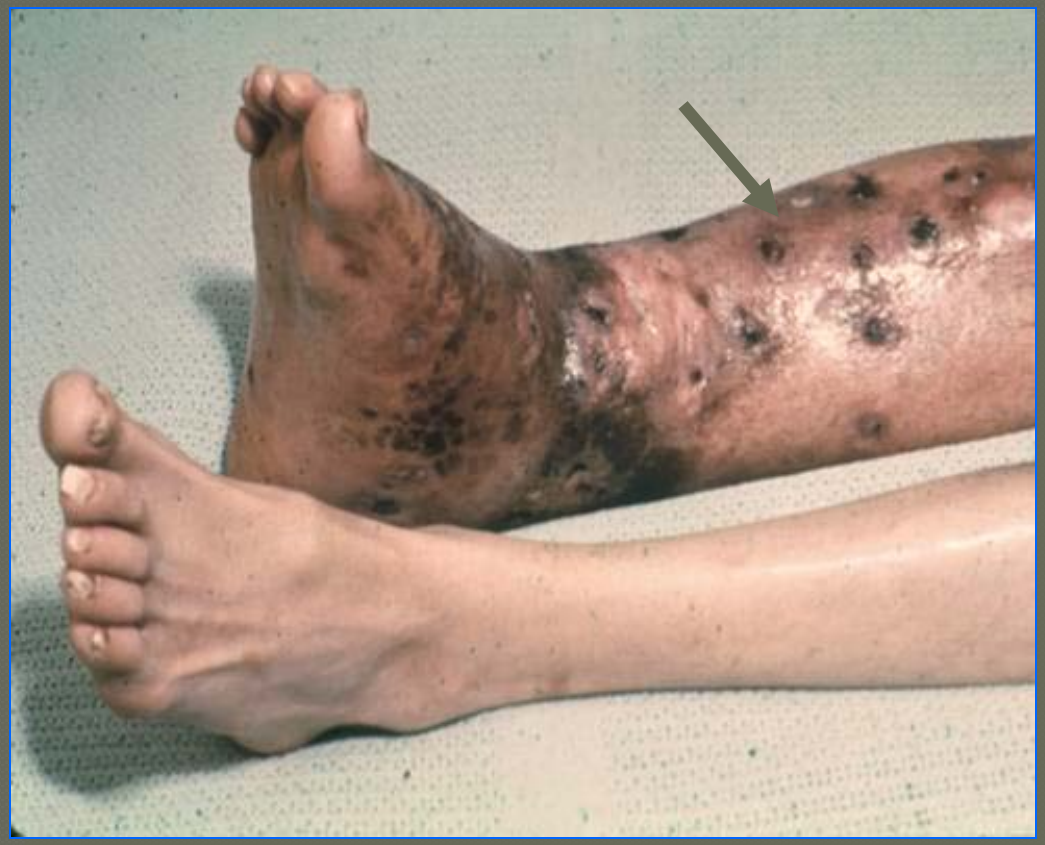
Multiple Staphylococcal Skin Abscesses

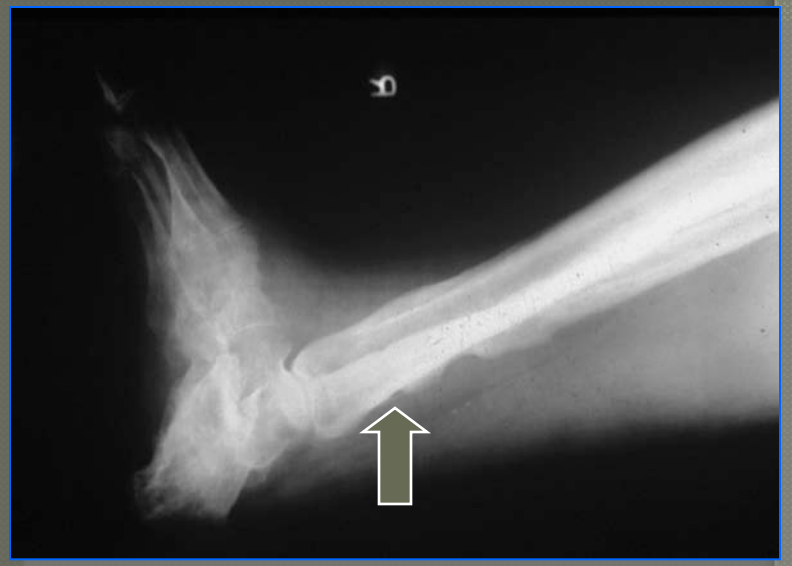


Foot Ulcer in a Diabetic Patient



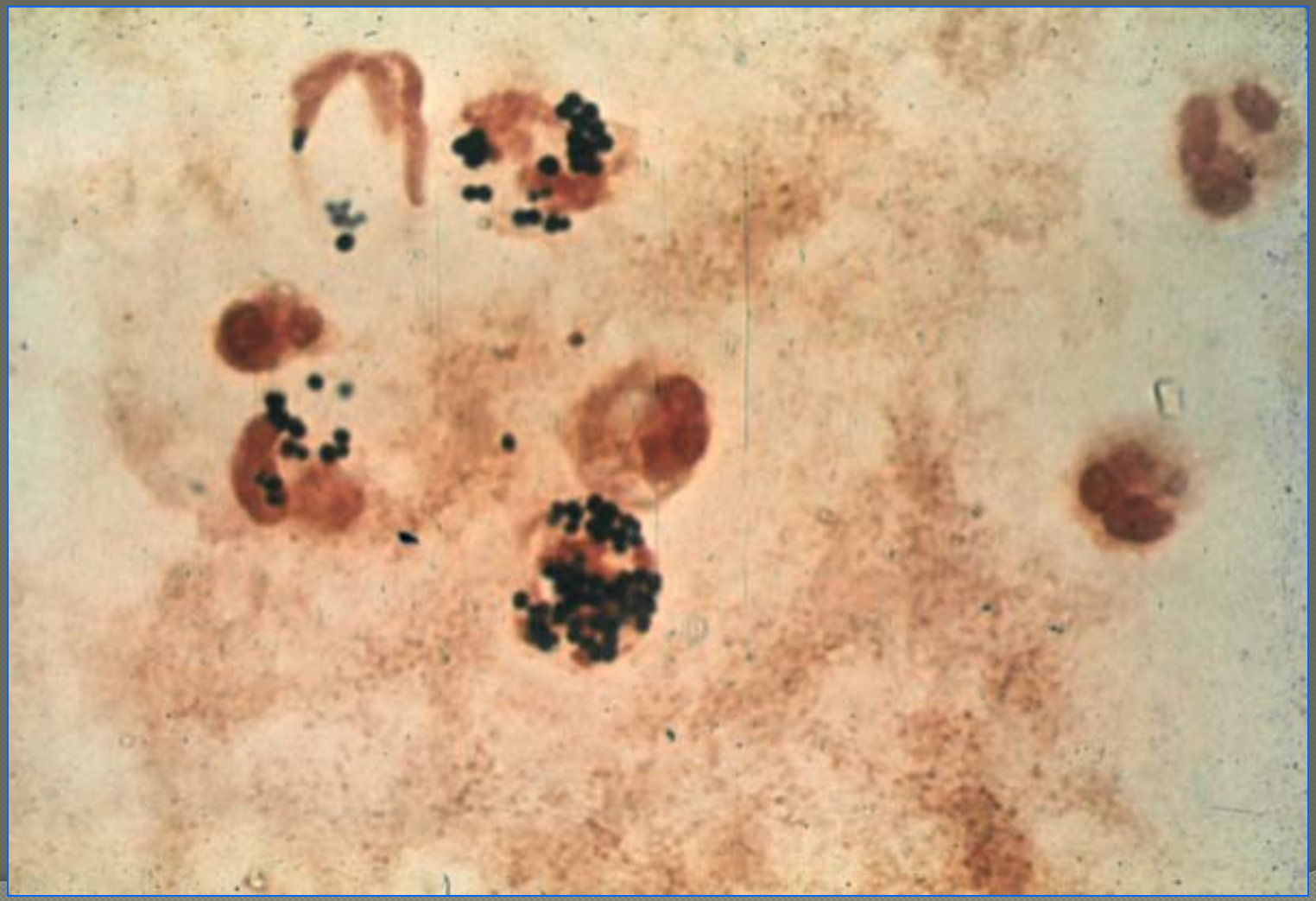
Staphylococcal Osteomyelitis and Drainage from Fistulae





Staphylococcal botryomycosis

Gram stained material from fistula



Resistance in *Staphylococcus aureus*

- Antibiotic resistance -- lots
  - Methicillin (oxacillin) resistance
    - Nosocomial
    - Community-acquired
  - Emerging resistance to vancomycin and other drugs used to treat MRSA.

MRSA

- First described in 1961; first penicillinase-resistant semisynthetic menicillin introduced in 1960.
- Acquisition of the *mecA* gene.
  - Codes for altered PBP; PBP2a
  - Variable expression
- Steadily increasing in nosocomial populations
  - Multi-resistant
- Community-acquired strains
  - Tend to be non-multi-resistant
  - Outbreak and sporadic
  - Skin & soft tissue infections

Detecting MRSA

- In clinical specimens
  - Gram stain and culture
  - PCR test for skin and soft tissue infections
  - PCR for rapid ID in positive blood cultures
- SURVEILLANCE
  - Increasing interest in detecting colonization
    - Primary site: anterior nares
    - Also axilla and other skin sites
  - Increasing data that detection & isolation of colonized patients can decrease infections
  - Specialized culture methods: 24-48h
  - Molecular testing: 1-2h

Molecular Methods for MRSA

- Sensitive and specific
- Rapid
- Relatively expensive
- Some are simple enough for POC use
  - None waived yet
  - Useful for rapid placement in surveillance
- Wound and soft-tissue infections
- (Blood culture rapid assessment)

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

The GeneExpert – Almost There!!!

- Self-contained molecular platform
  - Based on Smartcycler hardware
- Comparatively simple to operate
  - FDA-approved as a *moderate complexity* method.
    - Surveillance nasal swabs
    - Skin-soft tissue infections
    - Blood culture ID
  - In development as *FDA waived* method.

GeneExpert Testing

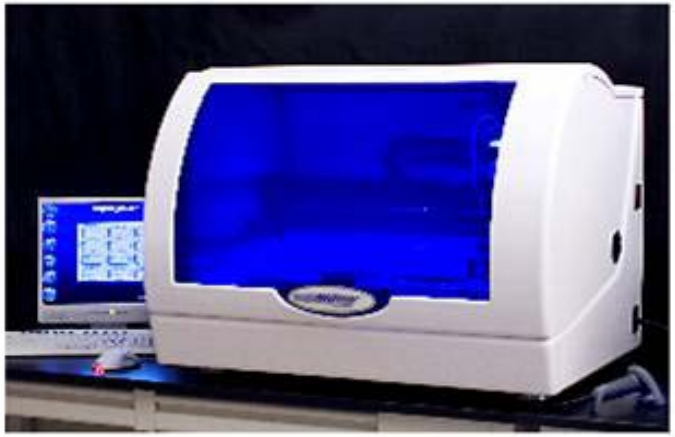
- Self-contained extraction / amplification / analysis
- Sample collected from nares on a swab
- Swab broken into extraction vial, vortexed, added to cartridge
- Reagents added
- Place cartridge in instrument, result in 60 min

Handylab 'Lab on a Chip'

- Small, low sample volumes, rapid analysis; combine
  - Fluid actuation
  - Sample pretreatment
  - Sample separation
  - Signal amplification
  - Signal detection
- To be distributed by BD

Handylab Systems

Jaguar



- Complete Automation
- Random Access
- Microfluidics
- Unitized Room Temperature Reagents
- Rapid Detection
- Contamination Control
- Open Menu for Laboratory Developed Tests

Raider

- Automated Amplification & Detection
- 12-15 Minute Real Time PCR
- Random Access
- Microfluidics
- Touch Screen
- Contamination Control

- 1-24 Samples
- 4 µl sample size
- 45-90 minutes
- Swabs, urine, plasma, csf, blood

Enigma Diagnostics FL and ML

- **Key features:**
  - Fully automated real-time PCR system
  - Rapid test (30 ~ 45 minutes to result)
  - Multi-sample and scalable accepts swabs and liquids (e.g. urine, blood plasma)
  - Integrated sample preparation and analysis
  - Low system price
  - Small footprint (no specialist skills or cold storage requirements
  - Miniaturized multi-well thermal cycling
  - Freeze dried reagents
  - Microfluid sample prep

IQuum

- Flexible tube divided into sealed segments

BioSeeq Plus Smiths Detection

- Handheld, fully field portable LATE PCR technology
- Supports up to 6 independent simultaneous assays
- Detects trace levels of specific BWA agents as low as 100 organisms
- Identifies biological warfare agents such as Anthrax, Tularemia, Plague and Pan-Orthopox
- Results in approximately 65 minutes
- Rechargeable lithium-ion battery pack

Specifications	
Size	30.5 x 18 x 7.5 cm (12 x 7 x 3 in.)
Weight	Detector (including battery pack) 3.0 kg (6.6 lbs) Battery pack 0.47kg (1.04 lbs)
Power	External: 12Vdc, via an ac adaptor Battery: Re-chargeable Lithium-Ion battery pack -can run up to 10 hours on a single charge
Communications	RS232. Can also be converted to USB with the addition of a commercially available serial connector
Capacity	Up to 6 independant simultaneously assays
Consumables	Self contained single use sample cartridge device PCR based training consumables available.
Typical Test Time	LATE PCR results in approximately 65 minutes depending on environmental conditions

Idaho Technologies Filmarray

- Automated Protocol; start the run & walk away
- Integrated Sample Preparation
- Automated analysis of results
- Results in less than an hour
- Microarray of up to 120 targets

What to think about

- 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

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."
- From "Clinical Microbiology in the 21st Century: Keeping the Pace". American Academy of Microbiology, 2008. Available on-line at: http://www.asm.org/academy/index.asp?bid=58445

Polling Questions

Acknowledgements

- Molecular platform slides courtesy Dr. Roger Klein.