

Update on Laboratory Diagnosis of Viral Infections

Jim Dunn, Ph.D., D(ABMM)
Cook Children's Medical Center
Ft. Worth, TX

How Far We've Come

- 1948 – cell culture techniques standardized
- 1956 – immunofluorescent stains
- 1960's – radioimmunoassays (RIAs)
 - electron microscopy
- 1977 – enzyme immunoassays (EIAs)
 - monoclonal antibodies
- 1983 – DNA hybridization probes
- 1985 – PCR

Goals of Testing for Viruses

Specific Viral Diagnosis (esp. rapid) Impacts:

- Duration of hospitalization
- Antibiotic usage
- Antiviral treatment/prophylaxis
- Number of other lab tests performed
- Infection Control/Public Health measures

“Rapid Viral Diagnostic Techniques”

1. Culture
2. Microscopy
3. Detection of Antigens in Clinical Specimens
4. Detection of Early Antibody Responses
5. Detection of Nucleic Acids

“Knowledge of test availability coupled with clinical information should lead to a diagnosis in sufficient time to influence patient management or treatment.”

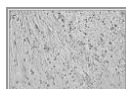
Chernesky, M.A. and J.B. Mahony. *Yale J Biol Med* 57:757 (1984)

Conventional Cell Culture

- need to use several cell lines
- examine for CPE
- hemadsorption (HAD) for Influenza, Parainfluenza
- minimum 10 days incubation
- requires refeeding media



Adenovirus (A549)



Influenza A (RMK)

Conventional Cell Culture

Virus	CPE	Time
Influenza	RMK	4 – 8 days
Parainfluenza	RMK	4 – 8 days
RSV	HEP-2/RMK	4 – 10 days
Adenovirus	A549	3 – 8 days
HSV	A549	1 – 4 days
CMV	MRC-5	10 – 30 days
VZV	A549	6 – 9 days

Leland, D.S. *Clin Micro Rev*, 20:49 (2007)

Cell Culture Methods

Advantages

- no false pos
- isolate range of viruses
- isolates for further characterization
- good sensitivity
- "gold standard"

Disadvantages

- need cell cx lab: equipment, personnel
- slower than Ag detection
- collection/transport conditions critical

Rapid Cell Culture

- shell vial format
- centrifugation-enhanced inoculation
- pre-CPE detection of viral antigens
 - fluorescent Ab stain at specified times
 - 24, 48, and/or 72 hours



Mixed Cell Culture Methods

- 2 cell lines per vial
- culture a broader spectrum of viruses
- eliminate need for multiple cell lines
- decrease reagent costs
- examine for CPE and/or viral antigens
- Respiratory: R-Mix (mink lung/A549)
R-Mix Too (A549/MDCK)



Sensitivity When Screening with R-Mix

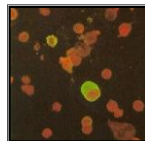
Virus	Total Pos Cxs	R-Mix Pos 24 hr	Avg. Time (d)
Influenza A	241	238 (99%)	1.9
Influenza B	38	36 (95%)	3.1
Parainfluenza 1	30	26 (87%)	3.8
Parainfluenza 2	2	2 (100%)	1.7
Parainfluenza 3	6	5 (83%)	3.7
RSV	60	52 (87%)	2.1
Adenovirus	51	35 (69%)	3.5
TOTAL	428	394 (92%)	2.3

n = 3,803 clinical specimens

Dunn, J.J. J Clin Micro, 42:79 (2004)

Direct Immunofluorescence

- several commercial products
- slides prepared from VTM pellet
- cytospin preparation
 - reduces number of inadequate samples
 - removes mucous
 - improves morphology & readability
 - easier to interpret



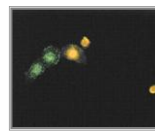
RSV DFA

Landry, M.L. J Clin Micro, 38:708 (2000)

Direct Immunofluorescence

SimulFluor Reagents (Millipore/Chemicon)

- direct immunofluorescence & culture confirmation
- dual color fluorescence: fluorescein & rhodamine
- FITC: antigens stain green or gold
- TRITC: only second antigen pink

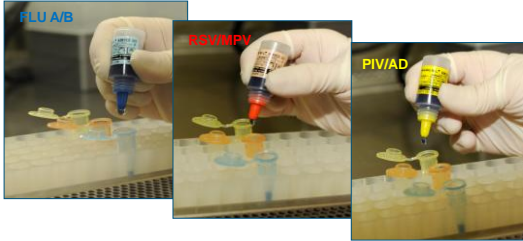


SimulFluor FluA/FluB

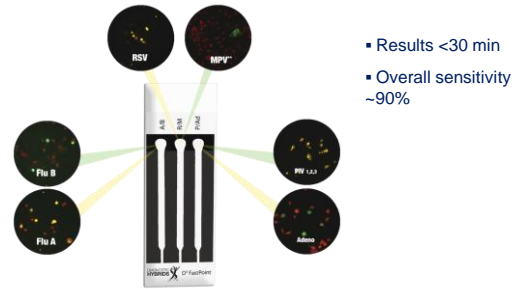
- Screen: RSV gold/others green
- Influenza A/Influenza B
- Parainfluenza 1,2,3/Adenovirus
- Parainfluenza 1,2/3
- RSV/Influenza A
- RSV/Parainfluenza 3
- RSV/hMPV

Direct Immunofluorescence

D³ FastPoint™ L-DFA Respiratory Viruses
(Diagnostic Hybrids)



Direct Immunofluorescence



Direct Immunofluorescence

Virus	Sensitivity	Specificity
Influenza A	50 – 90%	92 – 100%
Influenza B	45 – 88%	91 – 100%
RSV	81 – 100%	91 – 100%
Parainfluenza	45 – 80%	93 – 99%
Adenovirus	22 – 73%	91 – 99%
hMPV	63 – 99%	95 – 100%

Direct Immunofluorescence

Advantages

- rapid (30 min to 2 hrs)
- relatively inexpensive
- test all sample types
- batch testing
- assess specimen quality
- good sensitivity & specificity

Disadvantages

- high complexity
- subjective read
- fluorescent scope
- highly trained personnel
- longer TAT

Rapid Antigen Testing

Advantages

- rapid (10-30 min)
- amenable to POC
- moderate or waived complexity

Disadvantages

- expensive
- cannot assess specimen quality
- limited sample types
- subjective read
- less sensitive than other methods



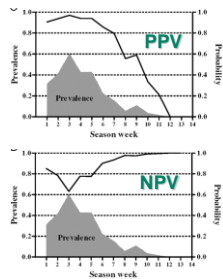
Rapid Antigen Testing

- Influenza A & B, RSV
- many commercial products available
- Sensitivities: 50% to 90%
- Specificities: 85% to 98%
 - as low as 50%
- better utility around peak months
- confirm early & late season positives
- back-up negatives with more sensitive methods?

Leland, D.S. Clin Micro Rev, 20:49 (2007)

Performance Characteristics of Rapid Influenza Testing

- children <5 yrs
- rapid influenza test
sens = 63%, spec = 97%
(compared to RT-PCR/cx)
- predictive value depends on prevalence



Grijalva et al. Pediatrics, 119:e6 (2007)

“Techniques Used in Diagnostic Virology”

G.A. Storch. Clin Infect Dis 31:739 (2000)

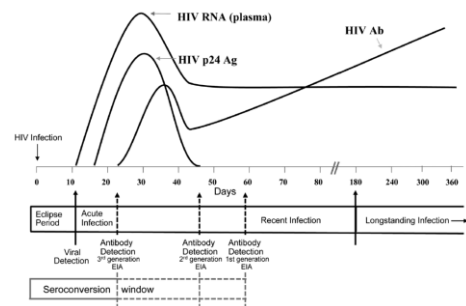
- cell culture
- antigen detection
 - fluorescent or immunoperoxidase Ab staining
 - enzyme immunoassay
- nucleic acid detection
 - PCR and other amplification methods
- microscopy
- serology

HIV Testing

CDC. MMWR 2006:55(RR-14)

- HIV screening for patients 13-64 yrs. in all health care settings
 - initiate treatment earlier
 - decrease transmission
 - in pregnancy, minimize transmission to newborn
 - occupational exposure, to interrupt transmission

HIV: Markers of Infection



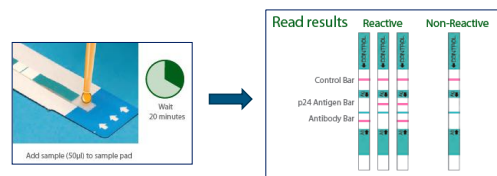
HIV Diagnostics

- HIV Ag/Ab Combo Assays
 - Abbott: FDA-approved June, 2010
 - Bio-Rad: FDA-approved July, 2011
- 4th gen assay also detects p24 Ag
- Identify infection 5-7 days earlier than 3rd gen EIAs
 - pos in 80% of NAT(+)/3rd gen(-) specimens
- 20 days earlier than WB

Pandori et al. J Clin Microbiol, 47:239 (2009)

HIV Diagnostics

- Alere Determine™ HIV1/2 Ag/Ab Assay
 - CE-marked for WB, serum, plasma



4th Generation Assays

- some demonstrate lower PPV than expected
- higher rate of false pos in low prevalence populations
- still need supplemental testing/confirm
 - +4th gen → EIA for HIV-1 vs. HIV-2
 - use NAT if HIV-1 & HIV-2 Abs neg

CLSI. Criteria for Laboratory Testing and Diagnosis of HIV; Approved Guideline M53-A (2011)

Molecular-Based Assays: Where We Stand

- Good for viruses that do not grow well
- For viruses we don't want to grow
- Labile viruses
- Overall increased sensitivity
- For labs without cell culture facilities or expertise in this area

FDA-Cleared Molecular Tests

Hepatitis Viruses

Assay	Manufacturer	Target(s)	Method
Real-time HBV	Abbott	HBV quant	qPCR
COBAS TaqMan	Roche	HBV quant	qPCR
VERSANT HCV	Gen-Probe	HCV qual	TMA
Amplicor HCV	Roche	HCV qual	RT-PCR
COBAS Amplicor	Roche	HCV qual	RT-PCR
Real-time HCV	Abbott	HCV quant	qRT-PCR
VERSANT HCV	Siemens	HCV quant	bDNA
COBAS TaqMan	Roche	HCV quant	qRT-PCR

FDA-Cleared Molecular Tests

HIV Assays

Assay	Manufacturer	Target(s)	Method
Real-time HIV-1	Abbott	HIV-1 quant	qPCR
NucliSENS HIV-1	bioMerieux	HIV-1 quant	NASBA
Amplicor HIV-1	Roche	HIV-1 quant	RT-PCR
COBAS TaqMan	Roche	HIV-1 quant	qRT-PCR
VERSANT HIV-1	Siemens	HIV-1 quant	bDNA

FDA-Cleared Molecular Tests

Human Papillomavirus

Assay	Manufacturer	Target(s)	Method
Cervista high risk	Hologic	14 types	Invader
Cervista 16/18	Hologic	HPV16 & 18	Invader
HC2 HR & LR	Qiagen	18 types	hybrid capture
HC2 HR	Qiagen	13 types	hybrid capture
COBAS HPV	Roche	16, 18, + 12 types	qRT-PCR

FDA-Cleared Molecular Tests

- NucliSENS EasyQ Enterovirus (bioMerieux)
 - CSF only/human Parechoviruses not detected
- Xpert EV (Cepheid)
 - CSF only/human Parechoviruses not detected
- BD ProbeTec Herpes Simplex Viruses (BD)
 - run on BD Viper platform (also CT/NG)
 - anogenital lesions only
- Multicode-RTX Herpes 1 and 2 (Eragen)
 - vaginal lesions only

Enteroviruses

Result	NASBA (n=59)	RT-PCR (n=59)	CSF Cx (n=59)	Stool Cx (n=53)
POS	35	37	22	30
NEG	24	22	37	23

Result	Xpert (n=138)	NASBA (n=60)	RT-PCR (n=138)
POS	25	22	25
NEG	111	36	113
Indeter.	0	1	0
Invalid	3	1	0

Marlowe et al. J Clin Virol, 43:110 (2008)

Dunn et al. CVS abstract T-AM20 (2006)

FDA-Cleared Molecular Tests

Respiratory Virus Detection

Assay	Manufacturer	Target(s)	Method
ProFlu+	Gen-Probe	FluA, FluB, RSV	qRT-PCR
ProhMPV+	Gen-Probe	hMPV	qRT-PCR
ProAdeno+	Gen-Probe	Adenovirus	qPCR
ProParaflu+	Gen-Probe	P1,P2,P3	qRT-PCR
ProFast+	Gen-Probe	A/H1,A/H3,pH1N1	qRT-PCR
xTAG RVP	Luminex	12 RVs	RT-PCR,Tags
Verigene RVP	Nanosphere	FluAs, FluB, RSVs	nanoparticles
FilmArray RP	Idaho Technology	394 (92%)	qRT-PCR
Simplexa	Focus Diagnostics	FluA, FluB, RSV	qRT-PCR
Simplexa	Focus Diagnostics	pH1N1	qRT-PCR
Xpert Flu	Cepheid	FluA, FluB, pH1N1	qRT-PCR

Molecular-Based Assays: Where We're Heading

- can we consolidate testing not just for virology but most (all) infectious diseases?

MICROFLUIDICS

MICROARRAYS

MASS SPECTROMETRY



Simplexa™



- centrifugal microfluidics coupled with rapid cycle real time PCR and fluorophore detection

GeneXpert®



- Self-contained, automated, fully integrated, random access instrument to detect bacteria or virus nucleic acid (DNA/RNA)
- Minimal hands-on time

Array Technology

- uses detection molecules ("probes") bound to solid surface to simultaneously detect multiple targets (e.g. DNA, protein)
- applications
 - gene expression
 - genotyping
 - pathogen detection
 - pathogen identification
 - sequencing

Luminex xTAG®

- suspension bead array coupled with flow cytometry-assisted detection
- versatile & flexible

Verigene® System

- 1° hybridization to glass slide array
- 2° hybridization to gold nanoparticle capture probes
- signal amplification
- other assays include:
 - cystic fibrosis
 - Factor II & IV
 - CYP2C9 & VKORC1

FilmArray®

The FilmArray Pouch

Future Plans?

GeneXpert	FilmArray	Simplexa	Luminex
HIV Quant	Bid Cx Gram +	EBV	xTAG GI Pathogen Panel
HCV Qnt/Genotype	Bid Cx Gram -	BK Virus	xTAG CYP2C19
HBV Quant	Bid Cx yeast	Enterovirus	xTAG CYP2C9+VKORC1
CMV Quant	Viral GI Panel	HSV 1 & 2	xMAP NeoPlex4
EBV Quant	Bacterial GI Panel	Grp A Strep	Allergy
RSV	Parasite GI Panel	Bordetella	Autoimmune
MTb/Rifampin	STD Panel	MRSA	HLA Testing
CT/NG	BioThreat Panel	<i>C. difficile</i>	
VAP MRSA/SA			
VAP Gram Neg			
BCR-ABL			
Cancer Genetics			

Mass Spectrometry for Virology?

PCR w/ electrospray ionization mass spectrometry (PCR/ESI-MS)

- coupling of broad-range PCR with electrospray ionization mass spectrometry
- molecular mass of PCR amplicons determined by MS and unique base (A,T,C,G) composition calculated
- base composition data from various primer sets compared to reference database to determine identification

PCR-ESI/MS

EXTRACT NUCLEIC ACIDS

↓

AMPLIFICATION WITH BROAD-RANGE PRIMERS, SPECIES-OR STRAIN-SPECIFIC PRIMERS

#	Mass	Base Count	Quantity
1	35875.03	A ₂₂ G ₃₄ C ₂₆ T ₂₆	4260
2	35297.70	A ₂₂ G ₃₃ C ₂₇ T ₂₅	1948
3	35819.87	A ₂₂ G ₃₄ C ₂₇ T ₂₄	1555
4	36196.21	A ₂₃ G ₃₇ C ₃₁ T ₂₆	1306
5	35297.70	A ₂₂ G ₃₃ C ₂₇ T ₂₆	1949

Base Compositions Map to Microbes

Utility of PCR-ESI/MS

- identification of bacteria, viruses, fungi, mycobacteria, parasites
- identifies complex mixtures of microbes (both expected and unexpected)
- resistance markers (mecA, KPC, vanA/B)
- high resolution genotyping/strain characterization
- no culture required, TAT ~6 hrs

Utility of PCR-ESI/MS

Rapid identification of viruses from nasal pharyngeal aspirates in acute viral respiratory infections by RT-PCR and electrospray ionization mass spectrometry. Chen et al., J Virol Meth 173:60 (2011)

RT-PCR/electrospray ionization mass spectrometry approach in detection and characterization of [influenza viruses](#). Deyde et al., Expert Rev Mol Diag 11:41 (2011)

Rapid identification of vector-borne [flaviviruses](#) by mass spectrometry. Grant-Klein et al. Mol Cell Probes 24:219 (2010)

Use of PCR coupled with electrospray ionization mass spectrometry for rapid identification of [bacterial and yeast bloodstream pathogens](#) from blood culture bottles. Kaleta et al., J Clin Microbiol 49:345 (2011)

Simultaneous identification of [mycobacterial isolates](#) to the species level and determination of [tuberculosis drug resistance](#) by PCR followed by electrospray ionization mass spectrometry. Massire et al., J Clin Microbiol 49:908 (2011)

Goals of the Past, Present, and Future

- Simplify Testing
- Minimize User-Required Steps
- Minimize Hardware Required for Testing
 - number and size of instruments
 - combine multiple functions on a single instrument/consumable
 - decrease footprint

What Does That Mean?

- less expertise needed to perform testing
- less lab space required
- increased portability
- POC or near-POC testing for real time diagnoses

