

Variola virus & smallpox: Past, present, or future tense?

- Orthopoxvirus Laboratory (DVRD/NCID/CDC)
- World Health Organization Reference Center for Orthopoxviruses

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Russell Regnery

Poxviruses (I)

- Large complex “brick shaped” virions
- Double stranded DNA
- Cytoplasm of host cells is the **ONLY** environment permissive for growth

Poxviruses (II)

2 Subfamilies:

Chordopoxvirinae (vertebrate poxviruses)

- **Orthopoxvirus (variola, cowpox, vaccinia, monkeypox, raccoonpox, camelpox, skunkpox, volepox, ectromelia, taterapox)**
- **Parapoxvirus (orf, pseudocowpox, ...)**
- **Avipoxvirus (canarypox, fowlpox...)**
- **Capripoxvirus (goatpox, lumpy skin disease...)**
- **Leporipoxvirus (myxoma, fibroma...)**
- **Molluscipoxvirus (molluscum contagiosum)**
- **Yatapoxvirus (tanapox, Yaba monkey tumor)**
- **Entomopoxvirinae (insect poxviruses)**

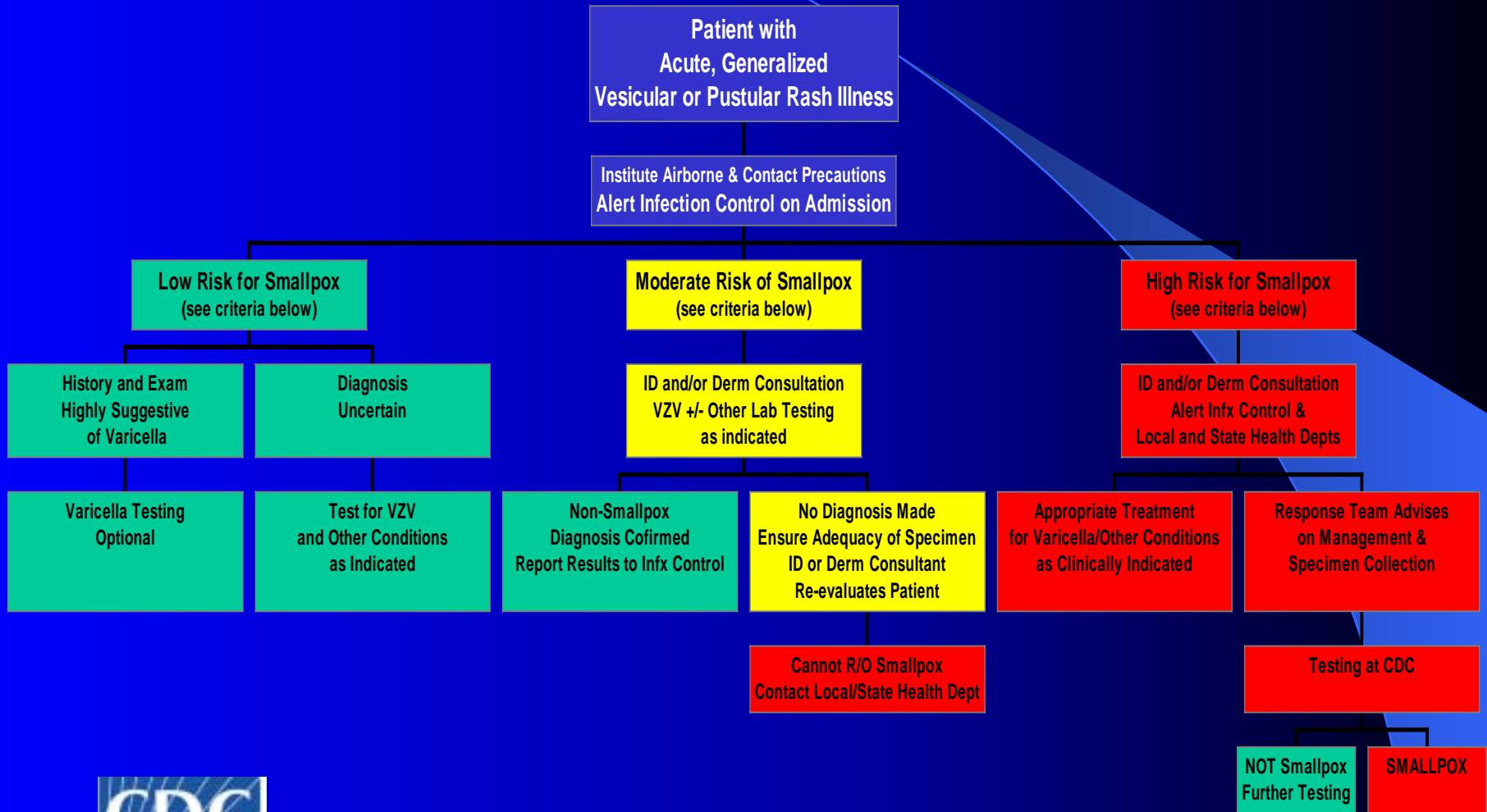
Orthopoxviruses: Spectrum of human disease in normal host

- Localized infections: vaccinia, cowpox
- Systemic illness: monkeypox, variola

vaccinia vs. variola

- **96% nucleotide identity**
- **Essential virion proteins with >98% AA identity**
- **Envelope (glyco)proteins important in humoral recognition over 93% AA identity**
- **Proteins predicted to, or demonstrated to be involved in immune evasion or host range demonstrate a greater range of homology**
 - **Variola encodes 24 ORFS whose Vaccinia homologs are truncated or absent**
 - **Vaccinia encodes 7 ORFS whose Variola homologs are truncated**

Febrile, vesicular rash illness algorithm



Diagnostic aims and goals

- Mitigate generation of false-positive results
- Provide laboratory capacity to confirm causes of febrile, vesicular, rash illness, including smallpox
- What combination of diagnostic(s) confers adequate sensitivity and specificity?
- How best to determine what is appropriate at the various levels (under different scenarios)?
 - “pre-event”, probability of smallpox is remote
 - “post-event”, vaccination would be implemented

Overview: laboratory methods for confirmation of Orthopoxvirus dx

- Virus culture
- Immunohistochemistry
- Electron microscopy
- Various PCR
- Serology
 - Antigen detection (IFA, EIA ag capture)
 - IgM capture
 - Neutralization antibodies **
 - IgG ELISA **

What about the Past? and Future??

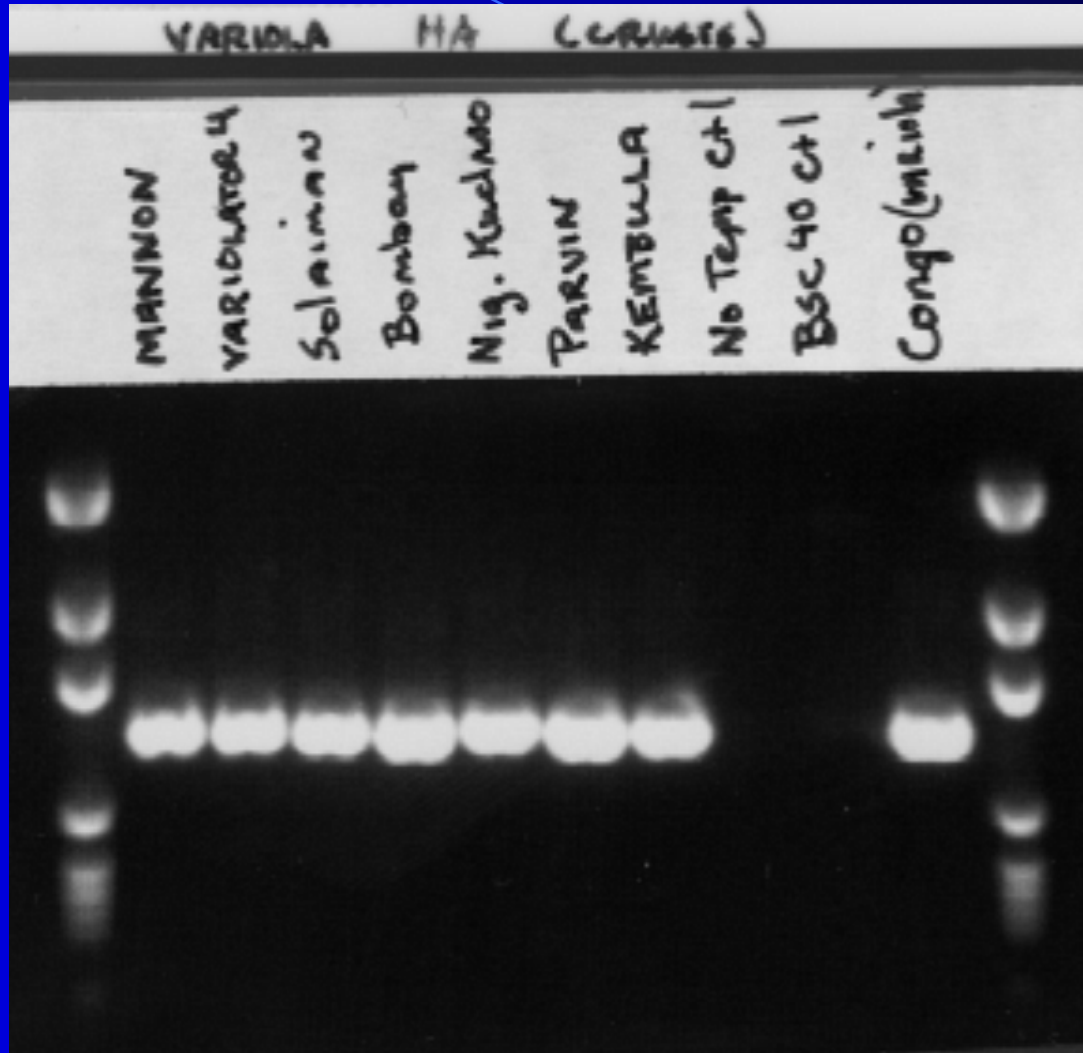
Virus Culture: The measure of infectious virus

- **The gold standard to which all measures of sensitivity are measured.**
- **Important source of reference material for analysis (e.g., detailed DNA analysis)**
- **If it isn't infectious, it isn't infectious (hoax scenario implications)**
- **N.B. Issues associated with culture as a diagnostic (facilities and treaty).**

PCR strategies

- **Essential, conserved genes (E9L, A25R)**
 - **Difficult to discriminate amongst species of orthopoxviruses (i.e. vaccinia and variola): typically species generic**
 - **Unlikely to be manipulated**
- **Nonessential, variable genes (HA, ATI, crmB)**
 - **Able to discriminate amongst species of orthopoxviruses: species specific**
 - **Sources of potential manipulation**

HA locus PCR: Variola scabs/crusts



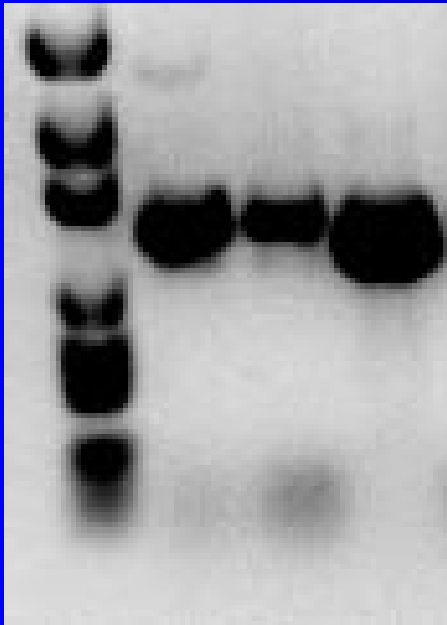
Sensitivity: 8/8

Correlates with > day 10 rash

PCR: Species generic

HA locus (5-6 hrs)

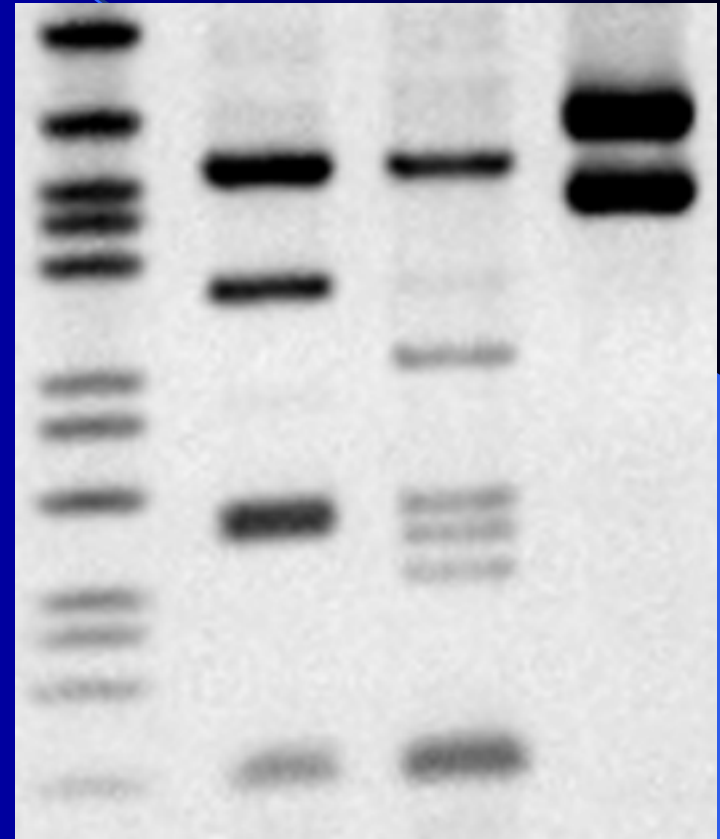
VAC MPX VAR



+ enzyme: *TaqI*

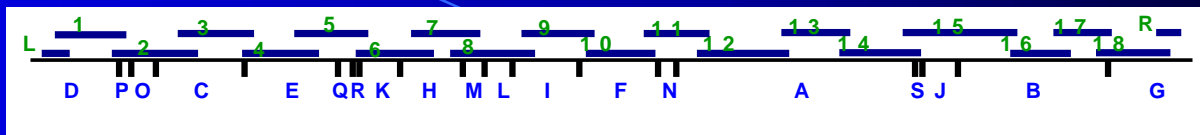
7-8 hours total

VAC MPX VAR



RFLP: Species specific

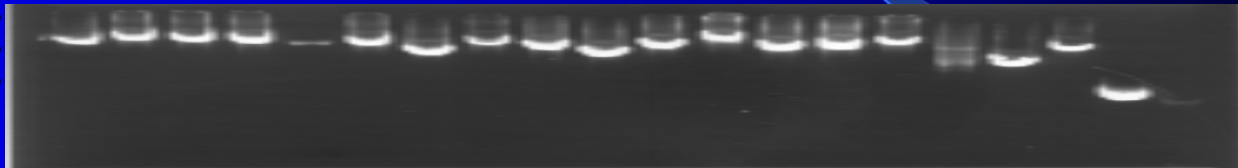
Extend-PCR/RFLP variola Bangladesh 1975



MW

PCR Amplicon

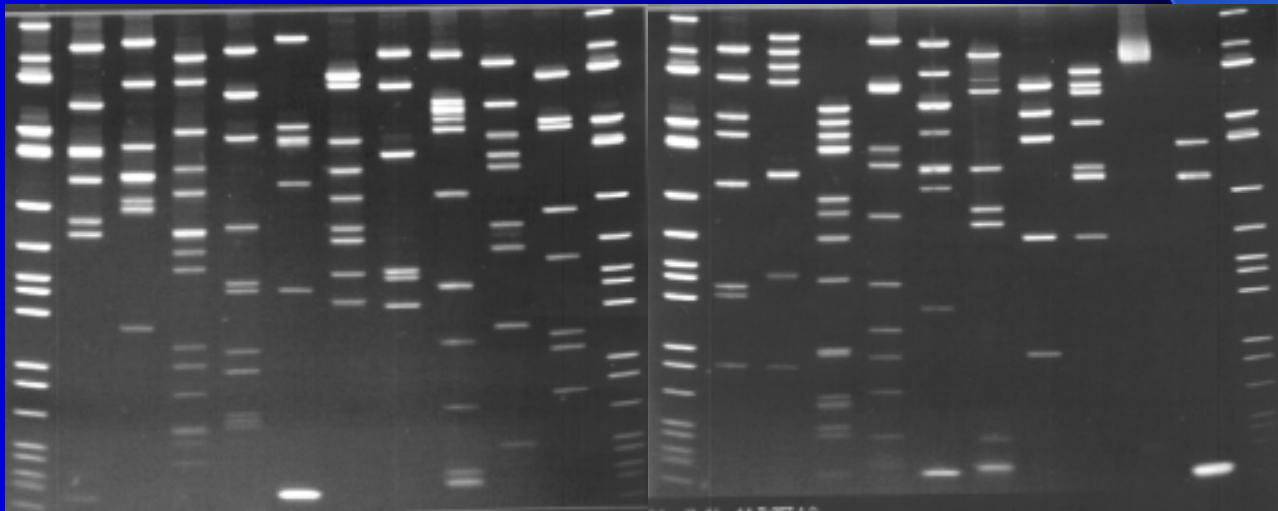
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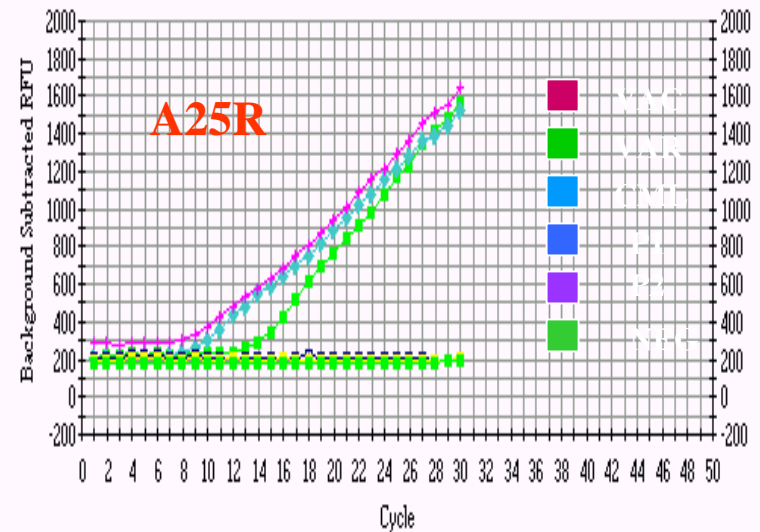
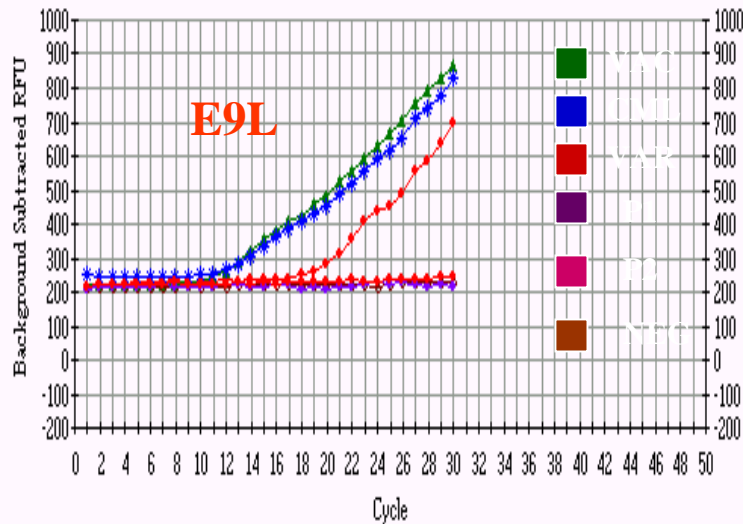
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*Bst*U I digest patterns

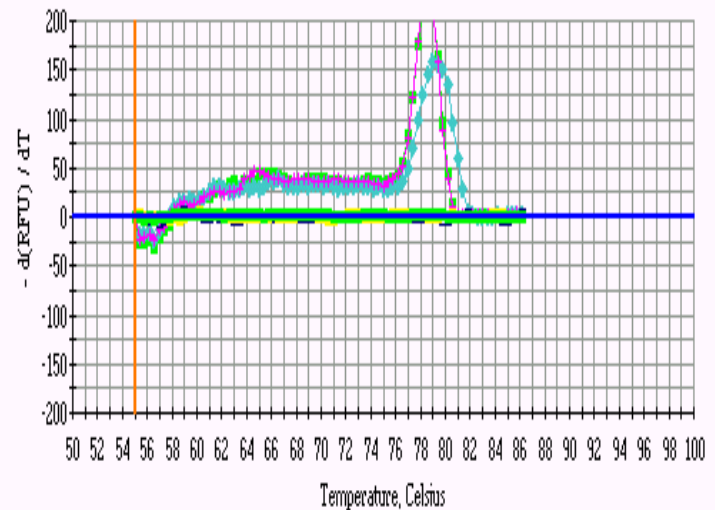
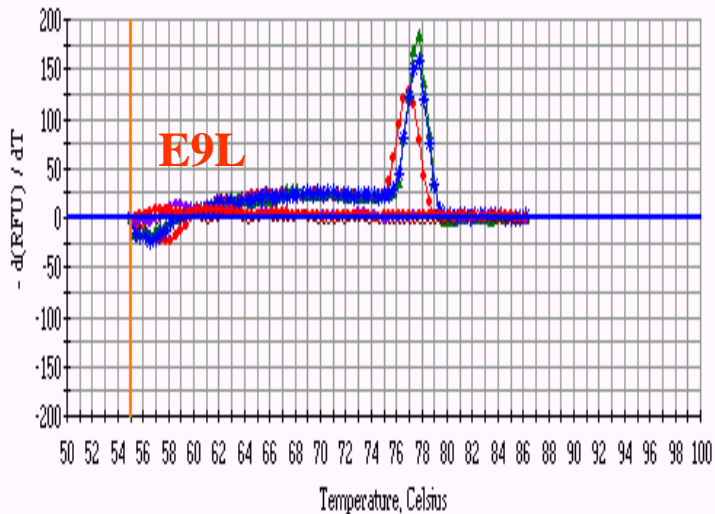
7087 bp
1605 bp
517 bp
222 bp
36 bp



Real time PCR of essential orthopoxvirus genetic locii: Rapid, high throughput screening test: NOT SPECIES SPECIFIC



Melt Curve analysis of PCR products



Real time PCR “TaqMAN” assay development

Essential gene target: E9L (DNA polymerase)

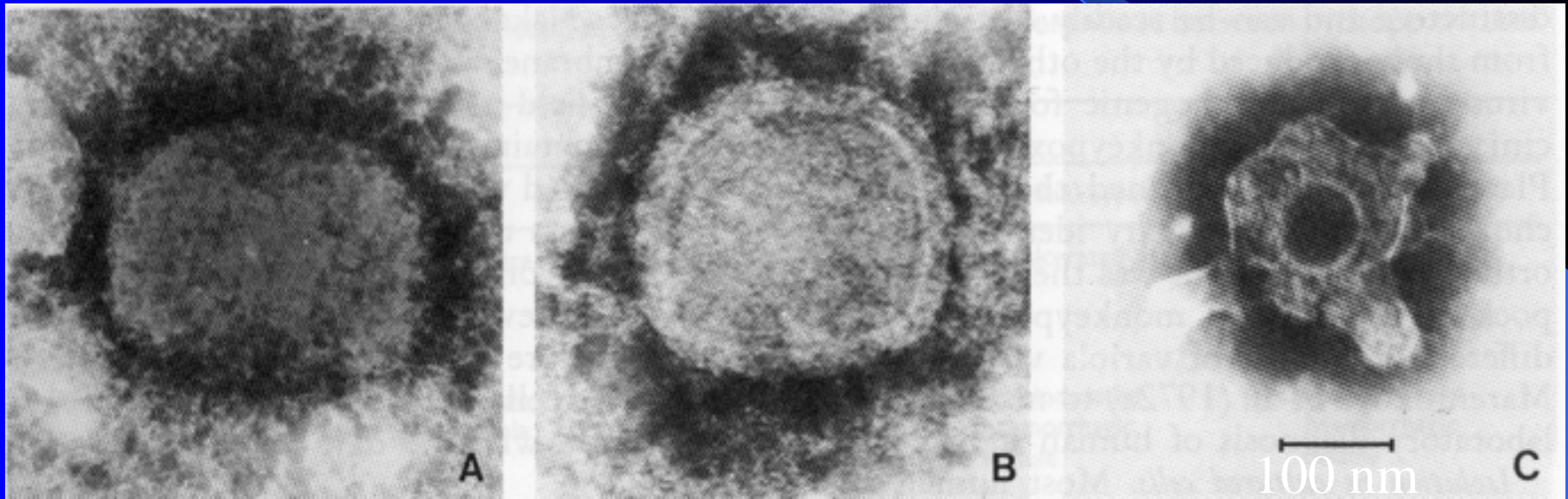
Variola SPECIFIC

monkey blood samples

Monkey#	d8 std (HA)	d8real time	d11std (HA)	d11real time	d14real tim
C083	Positive	Positive	Negative	Positive	Positive
C003	Positive	Positive			
57-394	Positive	Positive	Negative	Positive	Negative
C271	Positive	Positive (3039)	Negative	Negative	Negative
C282	Positive	Positive (2059)	Negative	Positive (290)	Negative
C835	Indeterm	Positive (647)	Negative	Negative	Negative
57-245	Negative	Positive	Negative	Negative	Negative
C677	Negative	Positive	Negative	Negative	Negative
C382	Indeterm	Positive	Indeterm	Positive	Negative
C409	Negative	Positive	Negative	Negative	Negative
48-48	Negative	Positive	Negative	Negative	Negative

Controls: monkeypox (10 to 10x10⁶ copies negative)
varicella (negative)

Negative stain electron microscopy variola vs. varicella

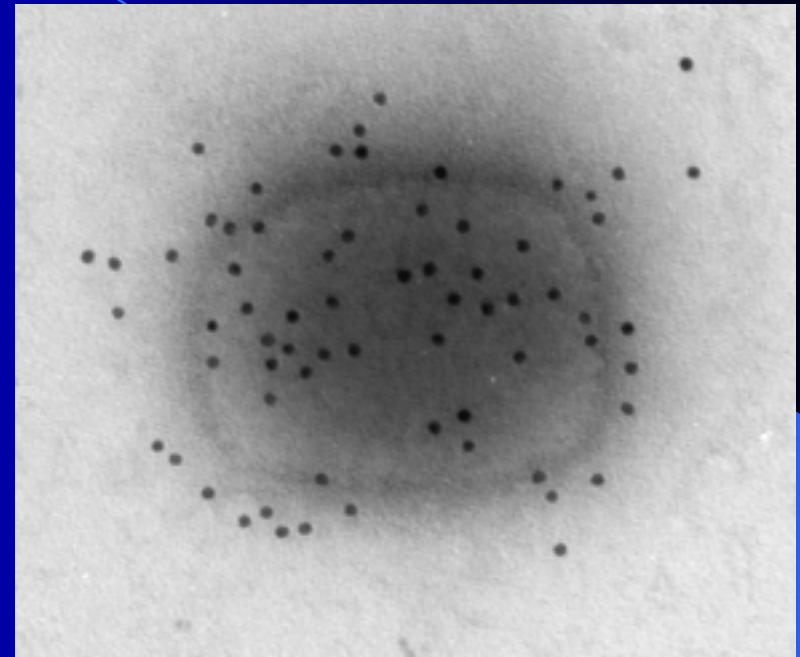
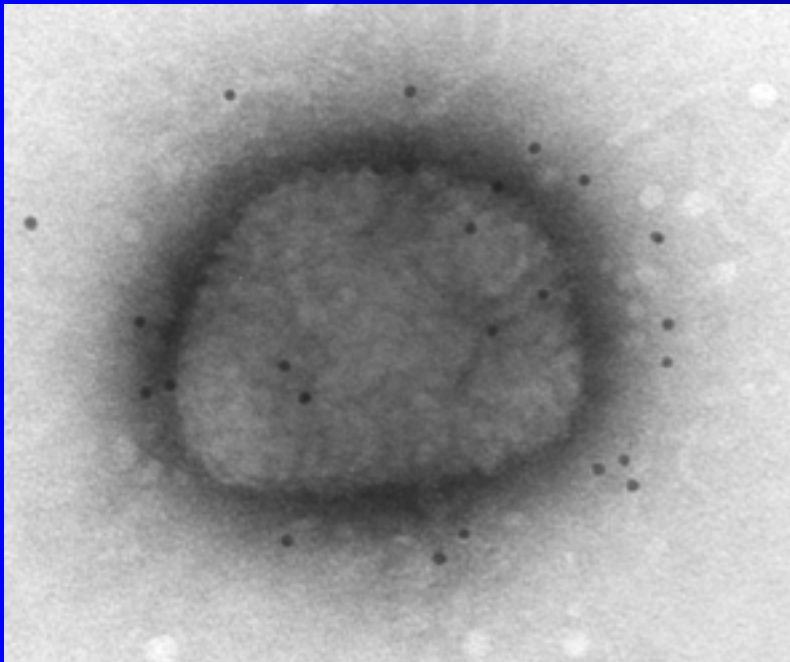


variola

variola

varicella

Immuno electron microscopy: orthopoxvirus (not species-specific)



Virus:	vaccinia	monkeypox
Primary:	1:5000 rabbit anti-variola	1:2000 mouse anti variola HMAF
Secondary:	12 nm colloidal gold conjugated , species specific	



Day 4 rash

Speciation of clinical sample by single gene PCR-RFLP analysis: ATI gene

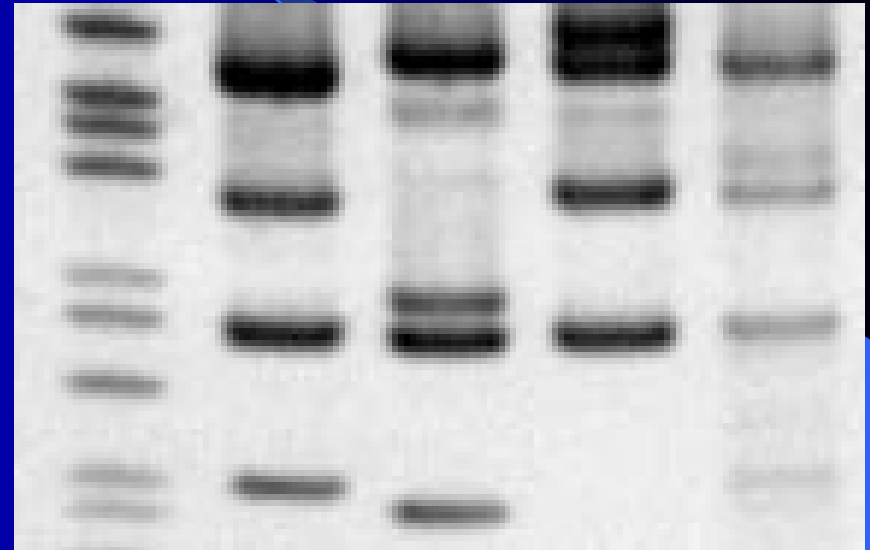
Case: v01-I-02

Specimens: vesicular fluid (v),
skin roof from vesicle(sk)

V M V S k v sk v v sk v sk v



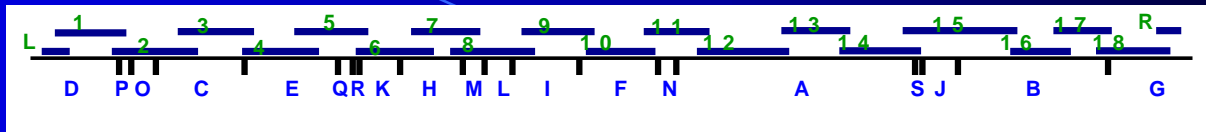
RFLP (BglII)
Vac Mpx Var Sk1



PCR amplified product yield: skin > vesicular fluid samples

1/8 amplicons sufficient yield for speciation by RFLP: Vaccinia

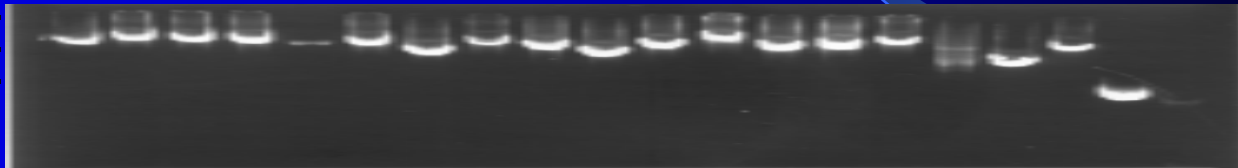
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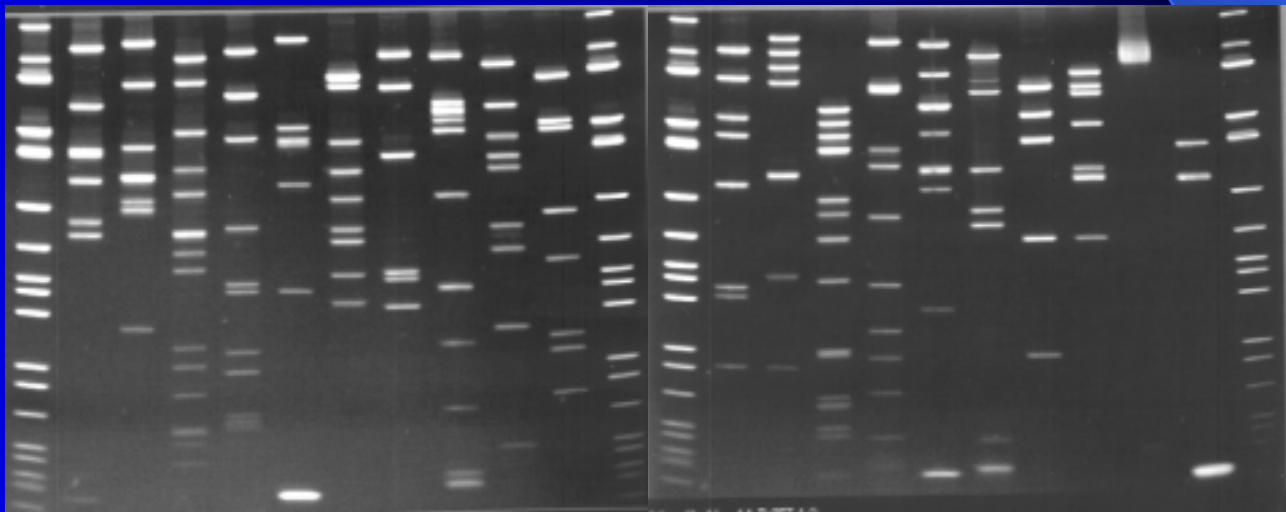
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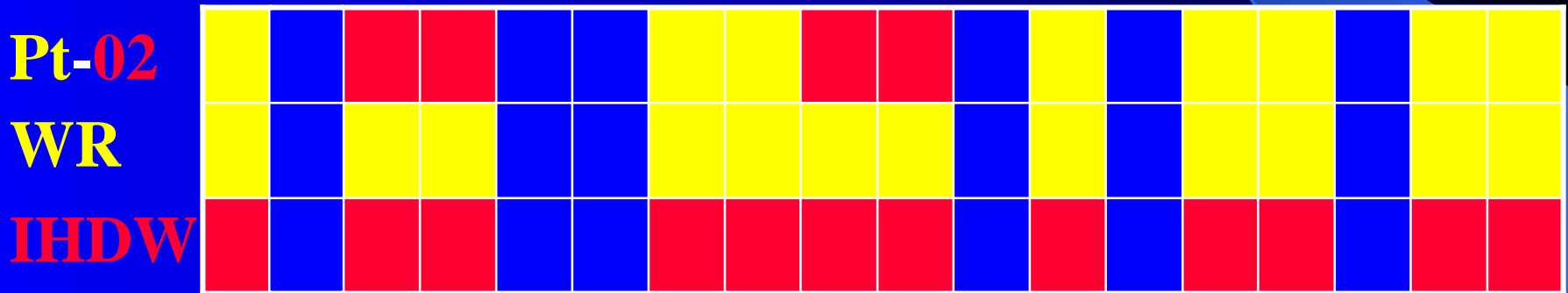
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**E-PCR RFLP evidence for multiple
crossover events to produce
Patient-02 isolate
from two common lab vaccinia strains**



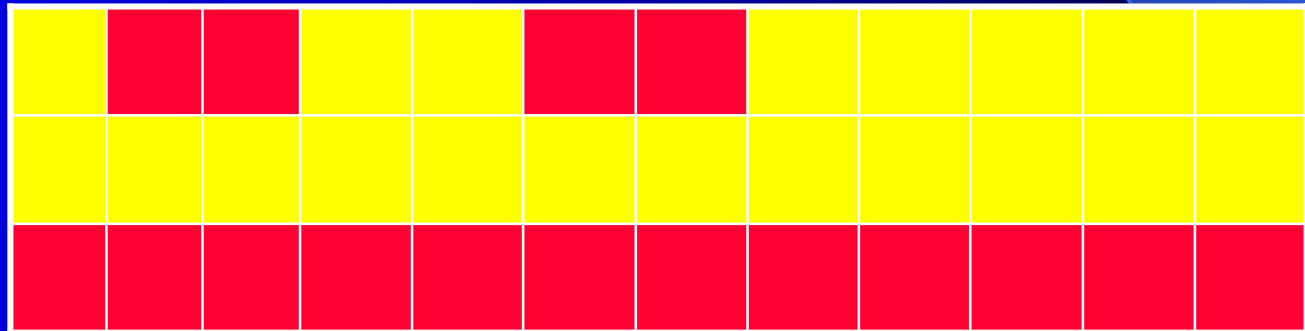
*Bst*UI digestion of amplicons 1-18

**E-PCR RFLP evidence for multiple
crossover events to produce
Patient-02 isolate
from two common lab vaccinia strains**

Pt-02

WR

IHDW



***Bst*UI digestion of amplicons 1-18,**

6 common band patterns not presented

Febrile vesicular rash illness example: disseminated vaccinia lessons summarized

- **DFA-VZV negative**
- **EM – no viral particles seen:**
 - **Optimize specimen collection: utilize grid to lesion method**
- **TaqMAN: correct answers in our lab**
 - **Need to standardize species specific assays, better characterize their sensitivity and specificity**
 - **Potential utility to screen for orthopoxvirus**
- **Orthopoxvirus IgM + at day 4 rash (first specimen)**
- **Culture positive <24 hours**
- **Interesting, intelligent answers possible**

Opportunities for pessimism

● “Dark optimism”:

- Smallpox was once an EID event (probably zoonotic) that subsequently benefited from thousands of years to evolve very clever mechanisms to optimize transmission and survival in a limited, host-specific, human context (prevaccination).
- Reminder of the need to be responsive to future possible EID poxvirus events (perhaps not completely unlike possible BT events).

● Sources for possible optimism:

- **Febrile Vesicular Rash Algorithm** is a creative way to enhance good medicine by better identifying smallpox look-alike diseases, and focus reference diagnostic resources on finite suspect smallpox cases.
- **A proven strategy for smallpox control exists (just in case).**
- **Considerable immune cross-reactivity (the basis of a proven vaccine). Implications beyond vaccines.**
- **Strategies for vaccines with less adverse rx's over the horizon.**
- **Increasingly “intelligent” analytic tests.**
- **Increasingly sophisticated understanding of basic orthopoxvirus virology leads to potential vulnerabilities.**