Prevention and Detection of Adventitious Infections of Mice with: Mouse Parvovirus (MPV) and Murine Norovirus (MNV)

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Prevention and Detection of Adventitious MPV and MNV Infections: **Seminar Overview**

- **Why use Specific Pathogen-Free (SPF) research models?**
- **How are animals kept SPF?**
- **Why are adventitious MPV and MNV infections still prevalent?**
- **MPV and MNV Surveillance**

Specific Pathogen-Free (SPF)

- Vast majority of animals used in research
- Test negative for:
  - Most or all known exogenous viruses
  - Pathogenic parasites
  - Limited list bacteria that may cause disease or otherwise interfere with research
    - Immunocompetent: Primary Pathogens
    - Immunocompromised: Opportunists

Why use SPF?

- **Adventitious (i.e., accidental) infections:**
  - Interfere with research
    - Disease
    - Contaminate biological materials
    - Subtle changes that alter:
      - Experimental responses
      - Phenotype in GM animals
  - Zoonotic agents pose a risk to public health
    - Subclinical in natural host
    - LCMV, Hantaviruses, *S. moniliformis*

Prevention of Adventitious Infections: **How?**

- **Adoption of Animal Production and Maintenance Processes that Emphasize Biosecurity**

Biosecurity

- All measures taken to:
  - Exclude (i.e., prevention)
  - Contain (i.e., limit spread)
  - Eradicate

  adventitious infections

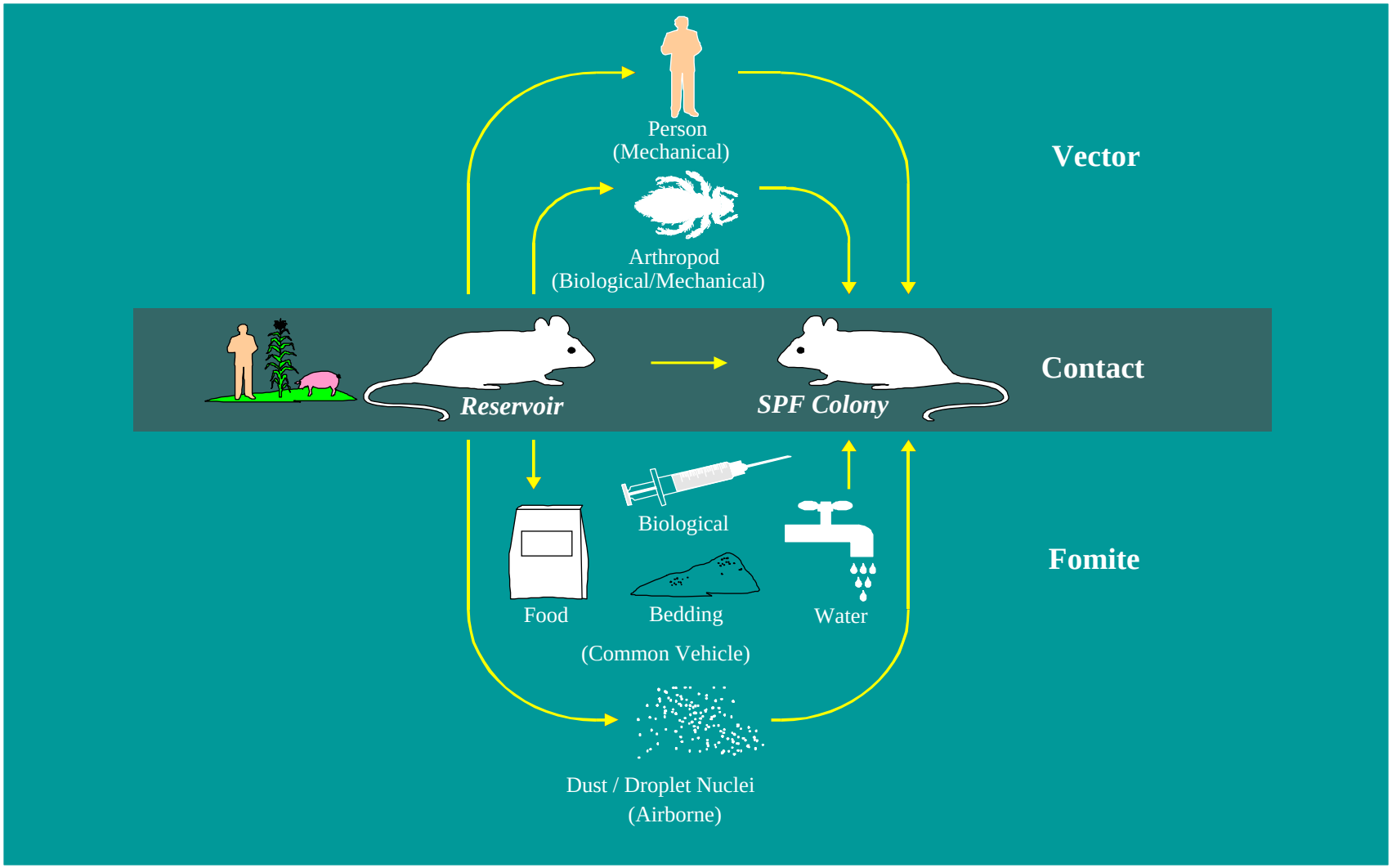
Biosecurity: Exclusion

- Simplistic approach is to look for “Smoking Gun”
- Appears more:
  - Expedient
  - Economical
- Often unproductive

Biosecurity: Exclusion

- Systematic Approach: Define all sources of infection and mitigate risk factors associated with those sources

Sources of Adventitious Infection



Sources of Adventitious Infection

- Shared Equipment

Biosecurity: Exclusion

- **Mitigate risk factors associated with sources**
- Direct Sources
  - Wild or escaped rodents: Pest control
  - Imported rodents: Active quarantine or rederivation
  - Utilize isolators or microisolators to contain and exclude adventitious infections

Biosecurity: Exclusion

- Is active quarantine necessary for vendor-supplied rodents?
  - Not for vendors from which you routinely receive animals
  - Caveats
    - Animals not shipped on dedicated transport may become infected in transit
    - Sentinels to be distributed among many rooms

Biosecurity: Exclusion

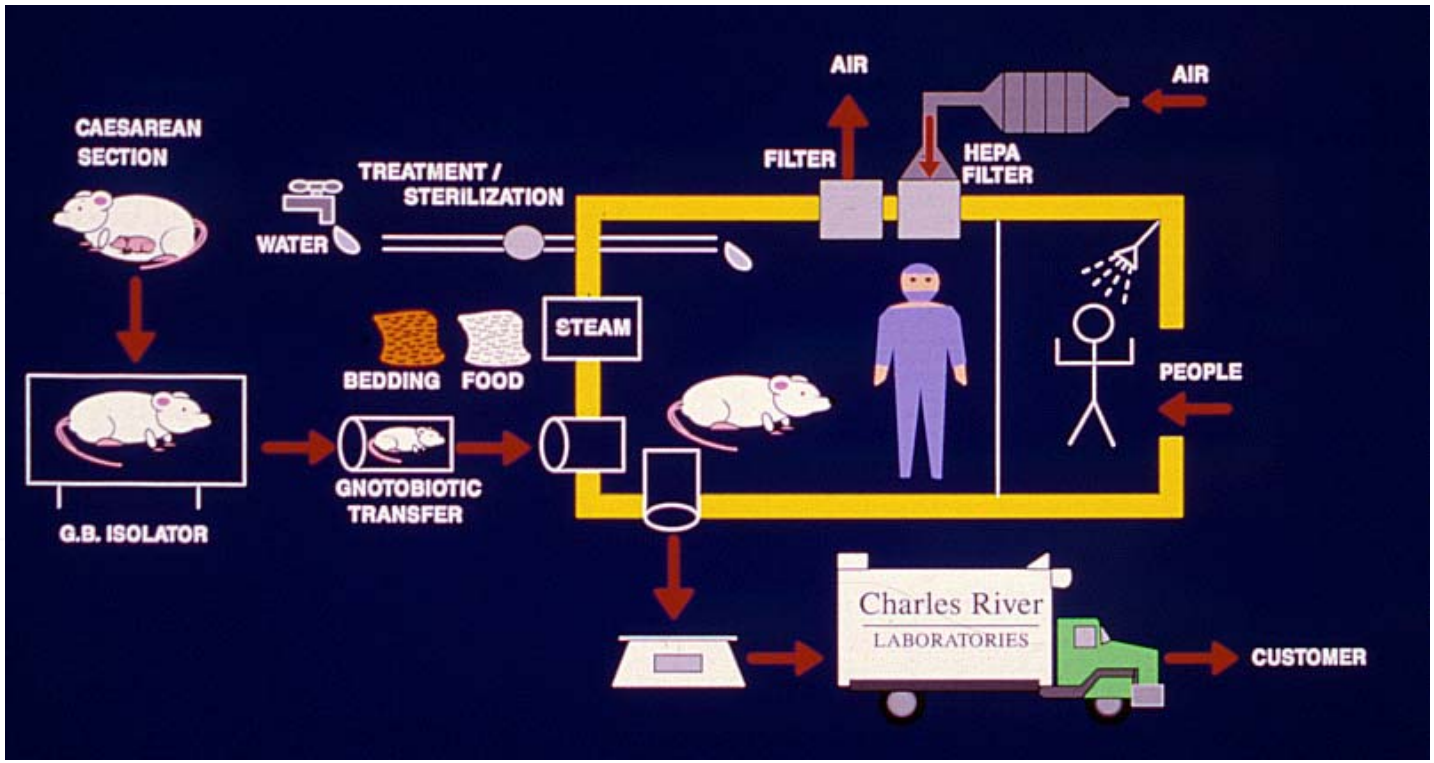
- **Indirect Sources**: Disinfection of Supplies and Shared Equipment
  - **Physical:** Autoclaving or gamma irradiation of food and bedding
  - **Chemical:** Chlorine disinfectants e.g., bleach and chlorine dioxide, of surfaces and water; ethylene oxide, H_2O_2 for equipment

Biosecurity: Exclusion

- **Indirect Sources: Personnel**
  - Gowning
  - Unidirectional workflow: moving from clean->dirty
  - Handling animals in laminar flow hood with disinfected gloves or forceps

Biosecurity: Exclusion

Cesarean-Originated Barrier-Sustained (COBS)



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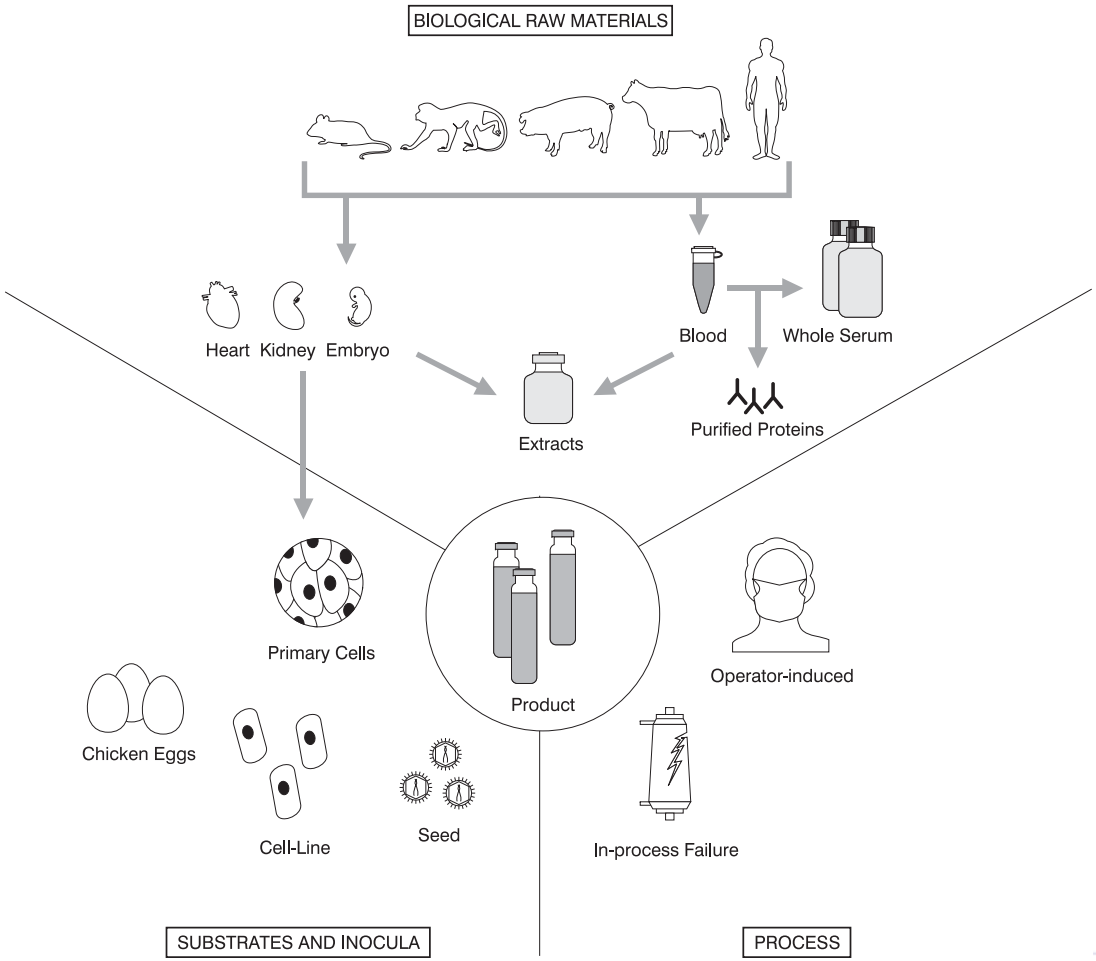
Biosecurity-Exclusion

- ## Indirect Sources: Biological Reagents

Virus[A]	Source	Reference
TMEV-TO	Yellow fever isolation	Theiler, M., 1937
PVM	Virus isolation in mice	Horsfall, F. L. and Hahn, R. G., 1940
K virus	MMTV stock	Kilham, L., 1952
Polyoma	MuLV stock	Gross, L., 1953
Sendai	Virus isolation in mice	Fukumi, H. *et al.*, 1954
MAV-1	Friend-MuLV stock	Hartley and Rowe, 1960
LDHV	Ehrlich carcinoma cells	Riley, V. *et al.*, 1960
RV	Rat sarcoma	Kilham, L. and Olivier, L. J., 1959
MTLV	Mouse tumor extract	Rowe and Capps, 1961
MVM(p)	Mouse adenovirus stock	Crawford, L. V., 1966
MVM(i)	Mouse EL4 lymphoma	Bonnard, G. D. *et al.*, 1976
MPV-1	T lymphocyte clone	Mckisic, M. D. *et al.*, 1993
MNV-1	RAG/STAT -/- mice	Karst, S. M. *et al.*, 2003

Sources of Contamination for Biological Reagents



Biosecurity: Exclusion

- **Indirect Sources: Biologicals**
  - Obtain **raw materials from reliable source** with certificate of analysis
  - Follow practices that **prevent operator-induced contaminations**, e.g., gowning and use of biological safety cabinets
  - **Bank** cell substrates and microbial inocula: Well characterized and standardized starting point
  - **Treat reagents** by physical (filtration, heat) or chemical (detergent, BPL) means **to remove or inactivate infectious agents**

Biosecurity: Exclusion

- **Indirect Sources: Biologicals (continued)**
- Testing for microbial contaminants particularly important for:
  - **Multiuse reagents and equipment**, e.g., chromatography columns
  - Cells and other biological reagents to be **inoculated into animal hosts**, which often produce much higher titers of an infectious agent than cell culture

Biosecurity: Elimination

- Rederivation By Hysterectomy or ET
  - **Most effective and methodology used at Charles River**
  - Eliminates yet-to-be-discovered agents
  - Expensive
  - Sacrifice valuable breeders
- Neonatal Transfer to SPF Foster Dam
  - Less expensive, saves breeders
  - Shown to be effective for eliminating *H. hepaticus* if done within 24 hours (Singletary et al, 2003)
  - Iodine Immersion (Watson et al, 2005)

Biosecurity: Elimination

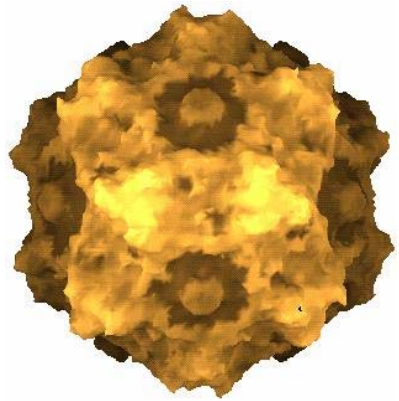
- **Moratorium on breeding** and the introduction of susceptible animals for enveloped viruses that cause non-persistent infections. Genetically modified animals may be immunodeficient.
- Antimicrobial Treatment
  - Successful on small scale for host-adapted bacteria that do no survive outside of host, e.g., *P. pneumotropica* and *H. hepaticus*
  - Parasite Infestations
  - Treatment has to be 100% effective
  - Extensive testing required to validate efficacy (i.e., cure)
  - May be toxic to genetically modified animals

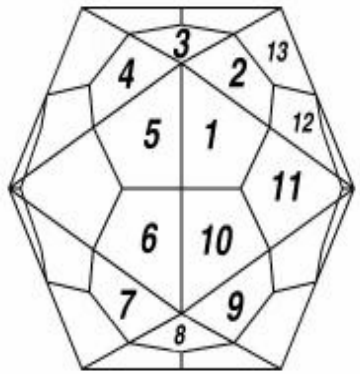
Efficacy of Laboratory Rodent Biosecurity

- Once common adventitious agents (e.g., Sendai virus, PVM, *M. pulmonis*) have become rare
- Exclusion and eradication of other agents has remained problematic, .e.g.,
  - Mouse Parvovirus (MPV)
  - Murine Norovirus (MNV)

Parvoviruses

- Molecular Biology
  - Comparatively small virus: approx 20nm
  - Non-enveloped
  - SS DNA genome of 5Kb
  - Proteins
    - Non-structural: NS1, NS2
    - Virion (coat) proteins: VP1, VP2
  - Replication requires mitotically active cells
- Predilection for dividing cells accounts for pathogenicity and research effects





Rodent Parvoviruses

- Serotypes
  - Mouse
    - Minute virus of mice (MVM, MMV)
    - Mouse parvovirus (MPV-1)
    - MPV-2, 3
  - Rat
    - Kilham rat virus (RV)
    - Toolan's H-1
    - Rat parvovirus 1 (RPV)
    - Rat minute virus 1 (RMV)
  - Newly recognized but not new
    - Retrospective MPV Serology: prevalent in mice over 30 years
    - Nonpathogenic: longstanding relationship with rodent hosts

*Newly recognized (since late 80's) with switch from HAI to ELISA/IFA

MPV Pathobiology

- **Nonpathogenic:** Even for neonatal and immunocompromised hosts
  Enterotropic: Fecal-Oral Transmission
- **Lymphotropic: Infection persists** in lymphocytes even seropositive immunocompetent mice
- Shedding NOT persistent in immunocompetent hosts

MPV Research Effects

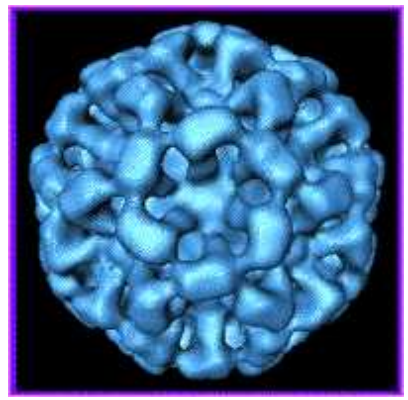
- Identification and propagation of a putative immunosuppressive orphan parvovirus in cloned T cells (McKisic et al, 1992)
  - CPE and erythrocyte aggregates observed in cloned murine T cell cultures
  - ↓ Proliferative response to IL-2 or antigen
  - Virus shown to be new parvovirus serotype
  - OPV->MPV-1a
  - Suspected sources:
    - MLC supernatant added as a source of lymphokines
    - Irradiated spleen feeder cells

MPV Research Effects

- MPV-1a Modulates Immune Response (McKisic *et al*, 1996)
  - Suppression of T Cell Response *In Vitro*
    - CD8+ T Lymphocyte Clones Lose Function and Viability
    - Cytokine- and Antigen-Induce T Cell Proliferation *In Vitro* Suppressed After Exposure to MPV-1a
  - Potentiates Allograft Rejection
  - Induces Isograft Rejection

Noroviruses

- Family *Caliciviridae* (*calix* = cup)
  - 28-35 nm
  - Non enveloped
  - Positive-sense ssRNA genome
    - ~7.4 – 7.7 kb with 3 ORFs
    - Single capsid protein: VP1
    - Various non-structural proteins
- Formerly referred to as Norwalk-Like viruses
- In people, causes >90% nonbacterial epidemic gastroenteritis worldwide; 23M cases/yr in US (*per* CDC)
- Five genogroups
  - I, II, IV: Human
  - III: Bovine (Jena)
  - V: Murine

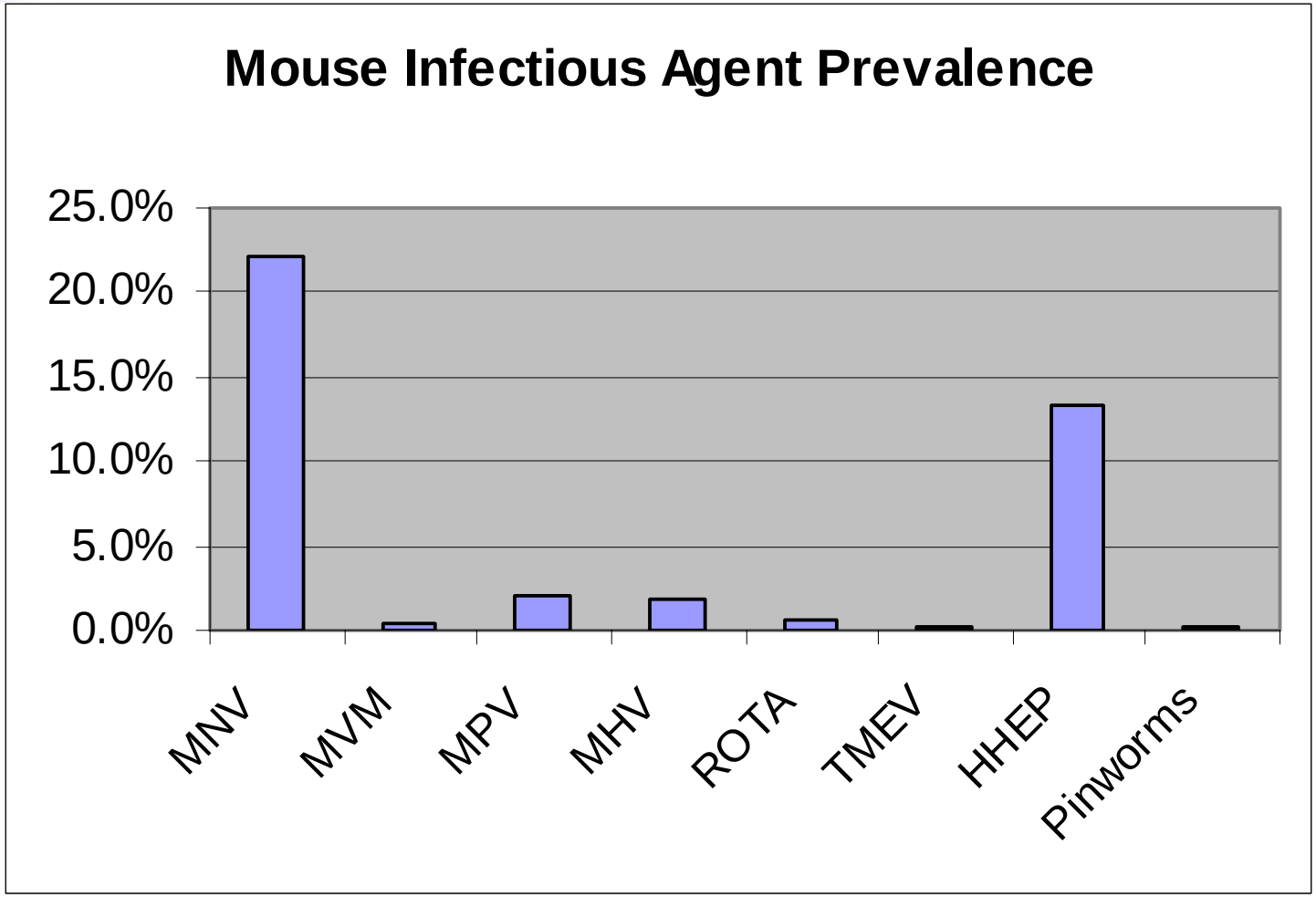


MNV Recently Discovered

- Reported by Research at Washington Univ starting in **2003**
  - Observed lethal disease in RAG/STAT1 double KO mice lacking innate immunity
  - Histopathology: Meningoencephalitis, cerebral vasculitis, hepatitis and pneumonia
  - Identified MNV-1 by representational difference analysis (RDA)
  - RT PCR: Virus detected in multiple organs and shed in feces
  - Tropism for macrophages and dendritic cells
  - MNV only norovirus propagated in a cell-line: Murine macrophage cell line RAW 264.7

MNV Pathobiology

- **Low pathogenicity:** Disease observed only in mice deficient in innate immunity
- **Shed in feces**, but gastroenteritis and diarrhea not reported
- **Infection and shedding are persistent** in immunocompetent hosts
- **Numerous strains exist**
  - Recombination (demonstrated for human noroviruses)
  - High rate of mutation: Lack of “proofreading” activity by RNA-dependent RNA polymerases
- **Widespread and prevalent**
  - RADIL: 22% by Serology, 30% by fecal PCR
  - Charles River RADS: 33% by Serology
- Host-adaptation, genetic variation and high prevalence indicate MNV **present for a long time**
- **Not in commercial breeder colonies**
  - Eliminated by hysterectomy and ET rederivation
  - Not vertically transmitted



MNV prevalence was reported by RADIL. Other data were from testing performed at Charles River Research Animals Diagnostics Services on non Charles River serum specimens

Reasons Why MPV and MNV Are Comparatively Prevalent

- **Only Recently Discovered**
  - Asymptomatic, benign, infections
    - Generally nonpathogenic *in vivo*
    - Difficult to propagate in culture (intentionally or inadvertently)
      - MPV: Only MPV-1a is cultivatable
      - MNV: Field isolates have to be adapted to RAW cells and initially produce little CPE
  - Before discovery, no surveillance
  - Inadvertently spread
- **Difficult to Exclude and Eradicate**
  - Infections are persistent: Contaminate biologics
  - Exceptionally stable, tiny non-enveloped viruses
  - Resistant to disinfection
  - Introduced via fomites: Notably food and bedding

MPV and MNV Surveillance

- Required because even the most rigorous biosecurity cannot be guaranteed to exclude all adventitious infections.
- Infections are inapparent.
- **Detected by laboratory testing**, referred to as **Health Monitoring** (or HM)

MPV and MNV Surveillance

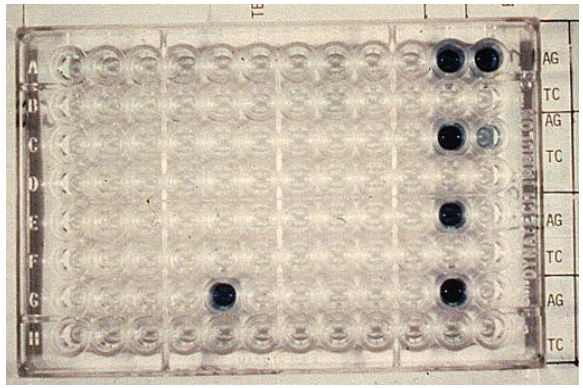
- **Surveillance Methodologies**
  - Disease and Active Infection
    - ~~Gross and Microscopic Examination: No Lesions~~
    - ~~Isolation: Many field strains are noncultivable~~
    - Detection of viral genomic sequence by PCR
      - Persists in host tissues following seroconversion
      - Stable in environment
  - Immune Response to Infection in Convalescent Host
    - Antibody Serology

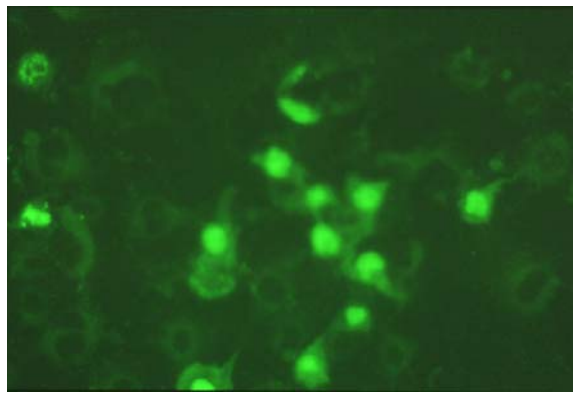
MPV and MNV Surveillance

- Serology: Primary surveillance technique
  - Despite generally persistent nature of parvovirus infections
  - Traditional, easy to do and inexpensive
  - Single serum simultaneously tested for antibodies to many agents
- PCR
  - Corroboration of serology: MLN, Fecal pools
  - Routine monitoring along with or instead of serology
  - Charles River: Quarterly MPV and MPV PCR on fecal pools

Rodent Serology

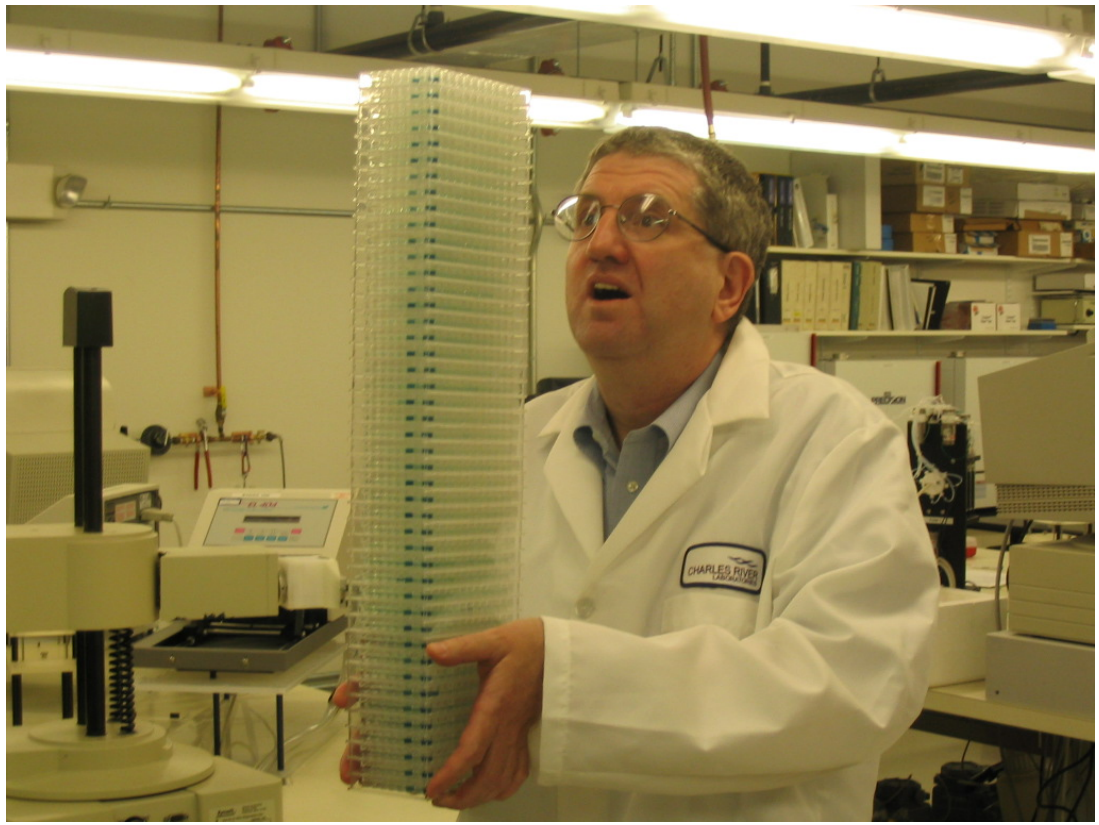
- Traditional: HAI/CF
- Supplanted by ELISA and IFA
  - **Screen by ELISA**
  - **Confirm by IFA**
- Performed as singleplexes, i.e., 1 assay reaction per test well





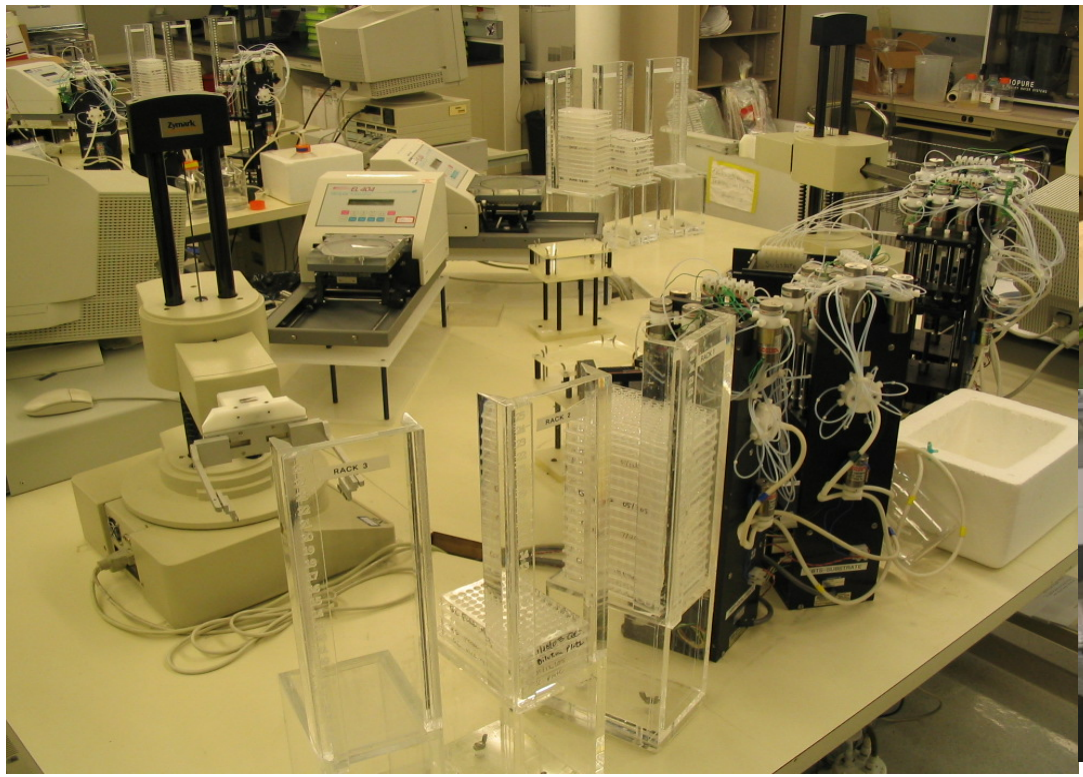
Issues with Large-Scale Testing by Singleplex ELISA

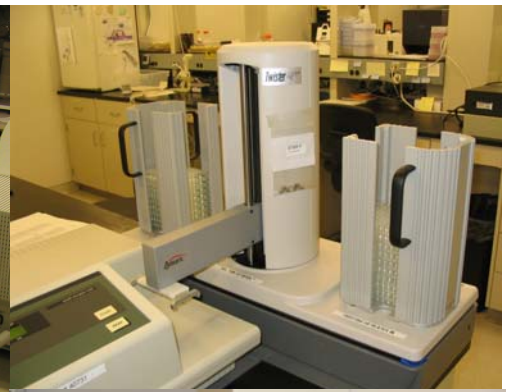
- Large stacks of plates: 800-1000 per week



Issues with Large-Scale Testing by Singleplex ELISA

- Complicated automation for plate and liquid handling



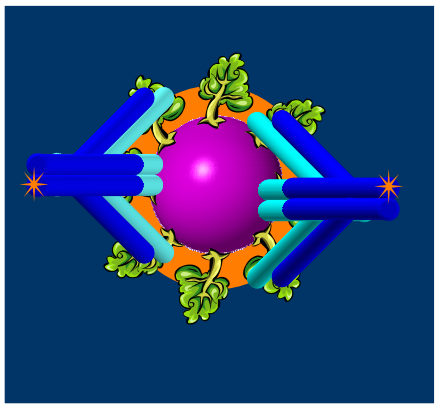


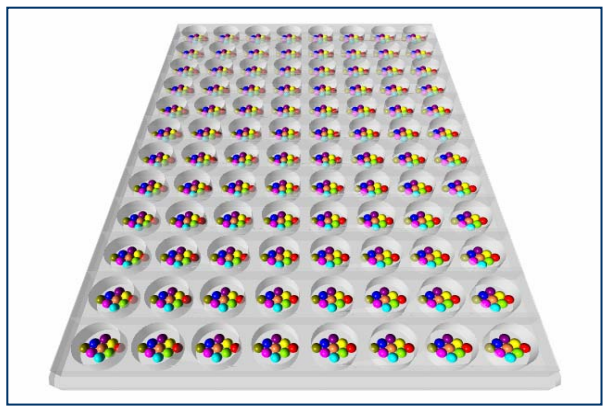
Issues with Large-Scale Testing by Singleplex ELISA

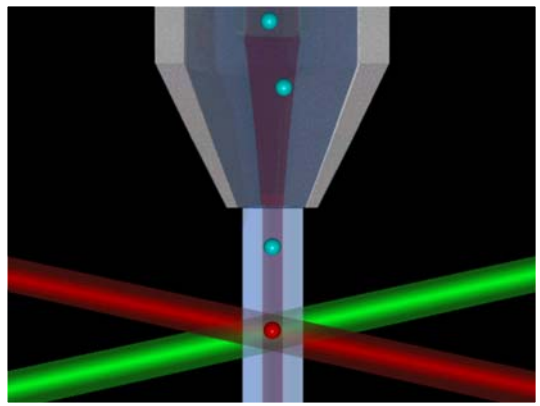
- Large liquid volumes: reagents and waste

Multiplexing: Multiple immunoassays performed simultaneously in a single well

- **Luminex Multi-Analyte Profile (xMAP)**
  - Suspension microarray: Antigen (capture antibody or NA probe) covalently linked to polystyrene beads
  - Beads in 100 color sets: up to 100 assays in single well
  - Detector determines bead color (i.e., assay) and reporter dye (phycoerythrin) fluorescence intensity one bead at a time, 25-100 beads per assay.
  - Intensity reported as median fluorescence index (MFI)
  - Multiplexed Fluorometric Immunoassay (MFIA™)

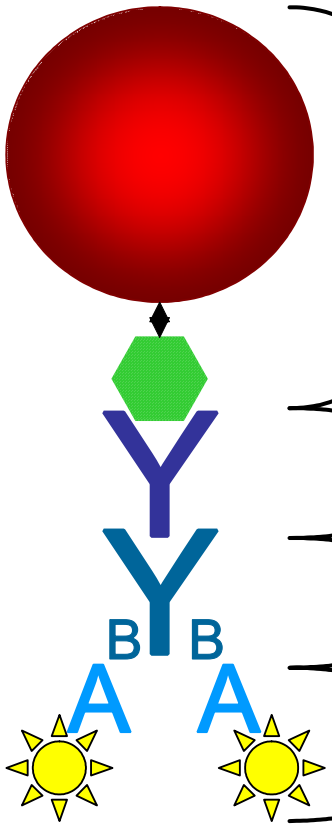






Fluorometer: modified flow cytometer

MFIA Procedure



Beads with Covalently Coupled (Conventional or recombinant) Antigen (PBS 2-3 hours at room temp)

Wash

Serum antibody (60 min)

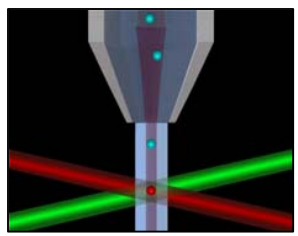
Wash

Biotinylated anti-species immunoglobulin (30 min)

Wash

Streptavidin-R-Phycoerythrin (SPE) Conjugate (30 min)

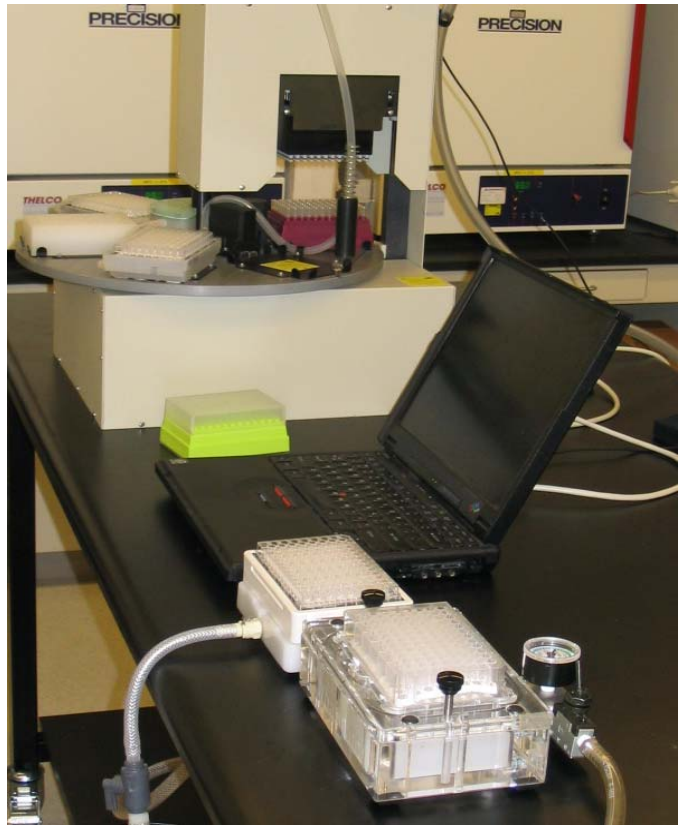
Wash



Read

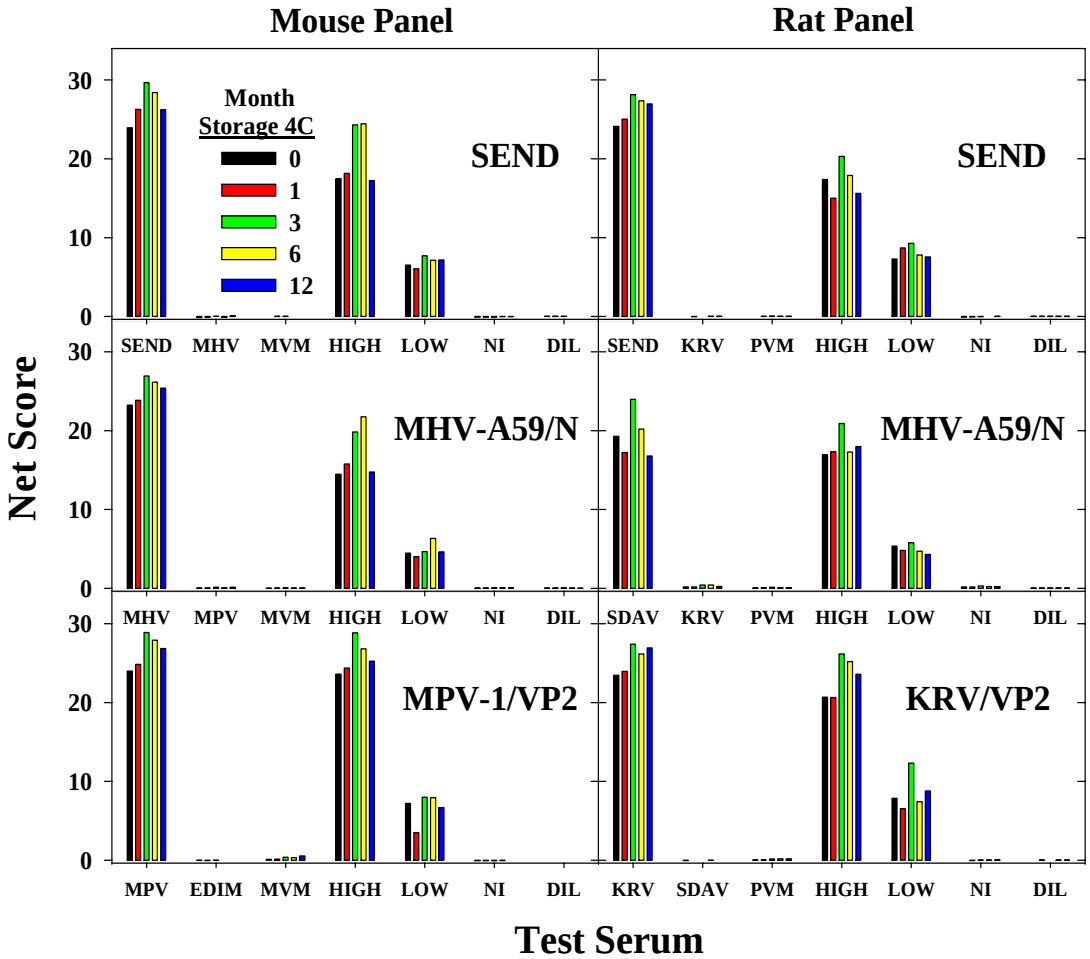
MFIA

- High throughput with much less
  - Liquid/reagent volumes
  - Plates
  - **Equipment**
- Passes KEEP IT SIMPLE STUPID (KISS) test

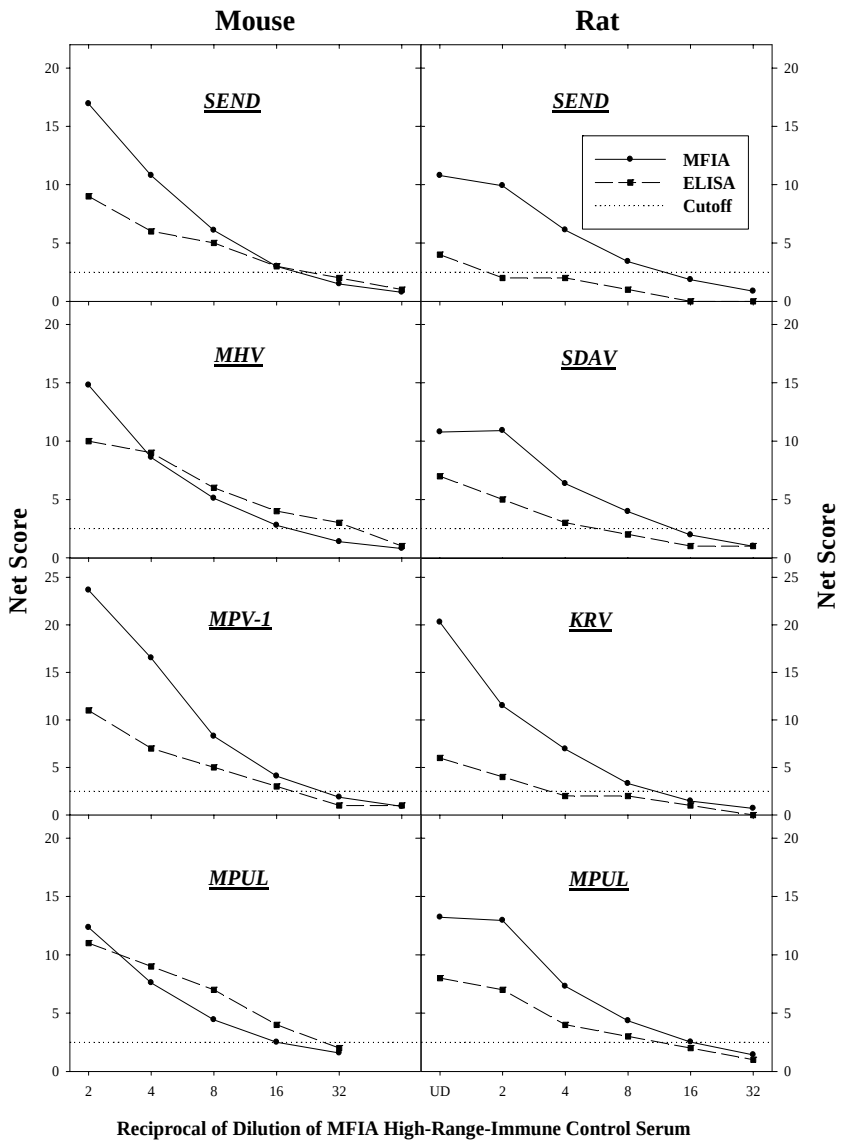


MFIA Validation: Analytical Performance

- **Stability** of MFIA Panel **Analytical Sensitivity and Specificity** during Storage at 4°C for 1 Year



Comparison of MFIA and ELISA Detection Limits



MFIA Validation: Diagnostic Performance

- Diagnostic Specificity for MFIA Performed on Known-Negative Sera from SPF Rodents

Classification	Mouse		Rat		Both	
	#	%	#	%	#	%
True-Negative	**416**	**100.0%**	**366**	**99.5%**	**782**	**99.7%**
False-Positive	0	0.0%	1	0.3%	1	0.1%
Borderline	0	0.0%	0	0.0%	0	0.0%
Nonspecific	0	0.0%	1	0.3%	1	0.1%
Total	416		368		784	

MFIA Validation: Diagnostic Performance

- Agreement between ELISA and MFIA Classifications of Known-Positive and –Negative Sera

Classification		Mouse		Rat		All	
ELISA	**MFIA**	**#**	**%**	**#**	**%**	**#**	**%**
+	+	154	22.9%	114	19.8%	268	21.5%
	-	6	0.9%	8	1.4%	14	1.1%
Sensitivity			**96.3%**		**93.4%**		**95.0%**
-	+	4	0.6%	26	4.5%	30	2.4%
	-	508	75.6%	428	74.3%	936	75.0%
Specificity			**99.2%**		**94.3%**		**96.9%**
Agreement		662	**98.5%**	542	**94.1%**	1204	**96.5%**

MPV Serology

- Parvovirus genome encodes nonstructural (NS1,2) and viral coat (VP1,2) proteins
- Antibodies formed to both NS and VP
  - **α NS**
    - NS proteins highly conserved; shared antigen
    - Cross-reactive
  - **α VP**
    - VP differ among serotypes, strains
    - Selective: Do not cross-react with heterologous serotypes, i.e., serotype specific
- NS antibody response can be absent or delayed
- To avoid false negative results, it is preferable to test for VP antibodies to each parvovirus serotype that naturally infects a rodent species

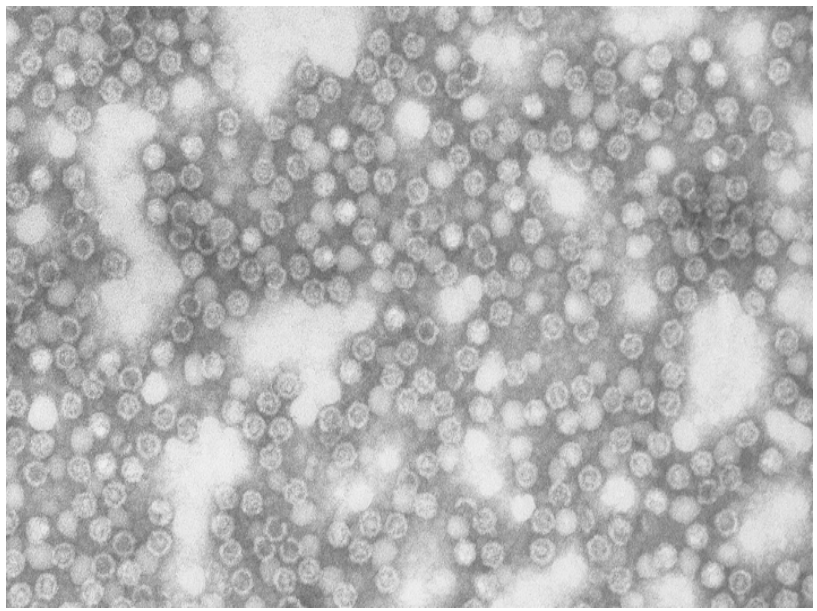
MPV Serology: Recombinant Antigens

- **MPV cannot be propagated in culture** to levels sufficient for preparing ELISA/MFIA antigen
- **MPV VP2 and NS1** genes inserted in baculovirus
- Expressed by infecting SF insect cell-line with recombinant baculovirus
  - Histidine-tagged NS-1 protein purified by Ni chelating chromatography
  - VP2 forms virus-like particles (VLPs), which can be purified by ultracentrifugation

MPV Serology: Recombinant Antigens

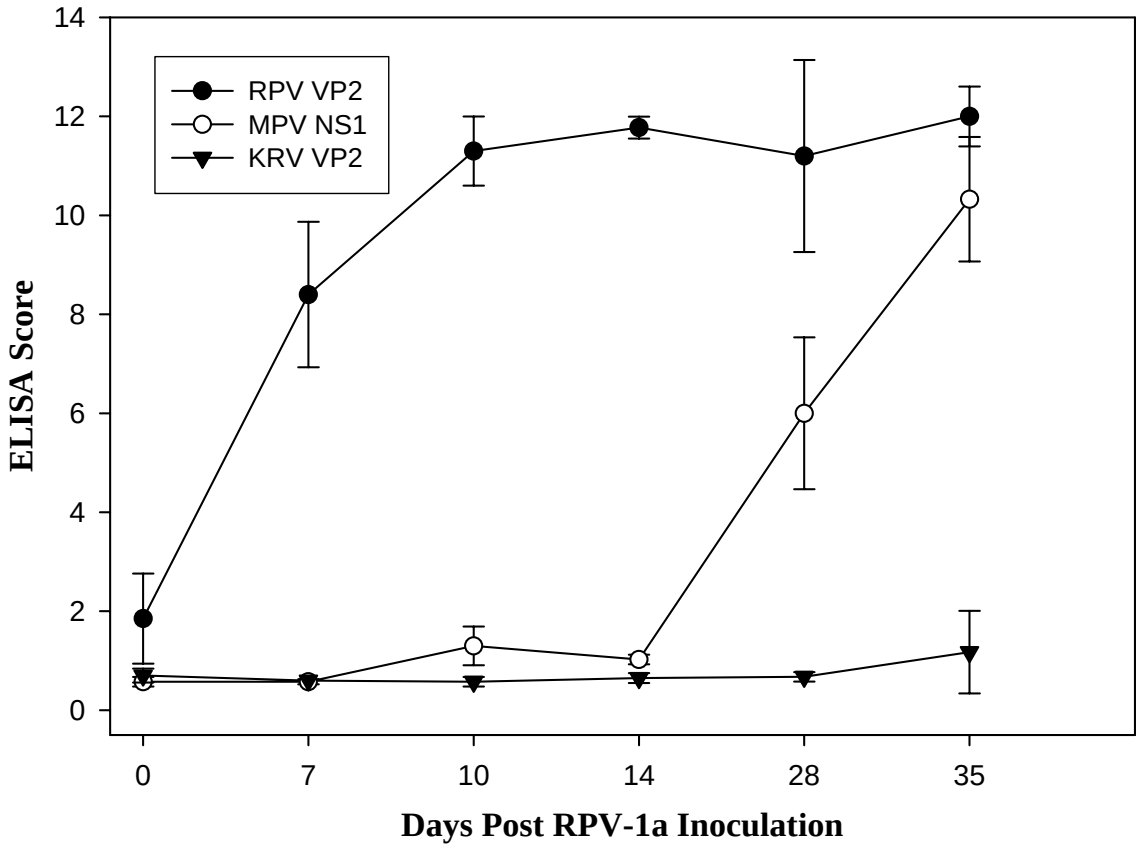
Native Parvovirus

Parvovirus rVP2 VLPs



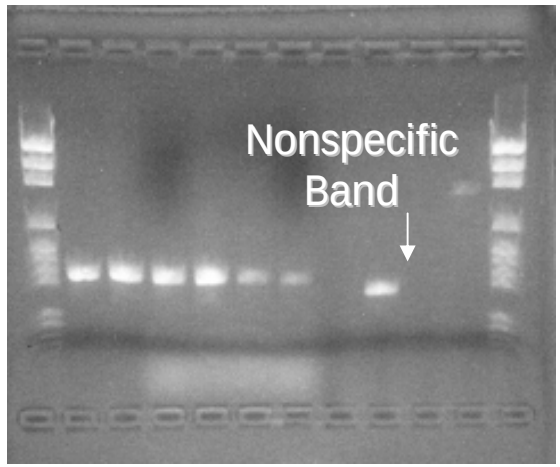
Parvovirus Serosurveillance

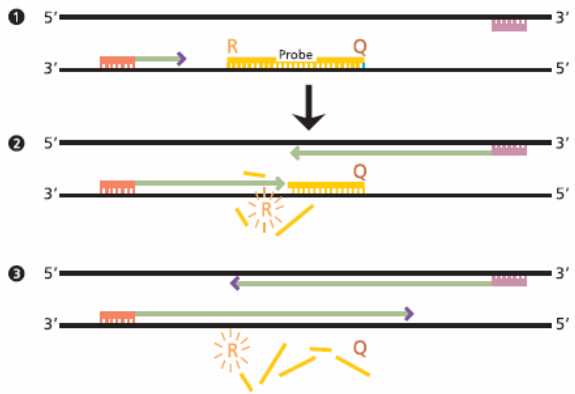
Comparative Serology on RPV-1a-Infected CD Rats



MPV PCR

- Assay Targets
  - Screening: Conserved NS1 Sequences
  - Serotype Identification: Unique VP2 Sequences
- Techniques
  - Gel-Based
  - Fluorogenic 5' nuclease TaqMan PCR used at Charles River
    - Incorporates hybridization of fluorophore-labeled probe
    - More specific than gel-based
    - More sensitive
    - Quantitative





MPV Surveillance: PCR and Serology Results Largely Agree

Table 4. Parvovirus serology and PCR results after an adventitious MPV infection of barrier-reared mice housed in uncovered cages[a]

Mouse strain	No. tested	No. positive	
		MPV-1 and -2 ELISA	NS1 PCR
BALB/cAnNCrl	32	17	17
DBA/2NCrl	8	1	3
CR Swiss	8	3	4
C57BL/6NCrl	8	0	1
B6D2F1	4	0	1
CB6F1	4	0	0
RFM/UN	8	4	4
All	72	25	30
Percentage positive		35%	42%

MPV Surveillance: Sentinels Sero- or PCR-Positive, but Study Mice Negative

- SPV (Sentinel Parvovirus) instead of MPV?
- **Sentinel results are false positive**
  - Laboratory Error
  - Infection of sentinels from extraneous sources
- **Low prevalence of infection**
  - Husbandry, e.g., microisolation cages in ventilated racks
  - Study animals less susceptible to infection than sentinels

MPV Surveillance: Sentinels Sero- or PCR-Positive, but Study Mice Negative

- Study animals less susceptible to infection than sentinels
  - Genetically modified mice are often on a C57BL/6 background
  - The dose of MPV required to infect C57s is higher than that for outbred stocks typically used as sentinels, e.g., Swiss Albino CD-1
    - Experience
    - Besselsen et al, Comp. Med.50:498-502, 2000

Quantification of MPV-1 Susceptibility of C57s vis-à-vis BALB/c (Pritchett et al, AALAS 2006)

- C57BL/6 mice are resistant
- 10 to 100 times more MPV-1 is required to infect C57 than BALB/c Mice
- When infected, C57s become both PCR and seropositive
- Do not use C57s as serology sentinels

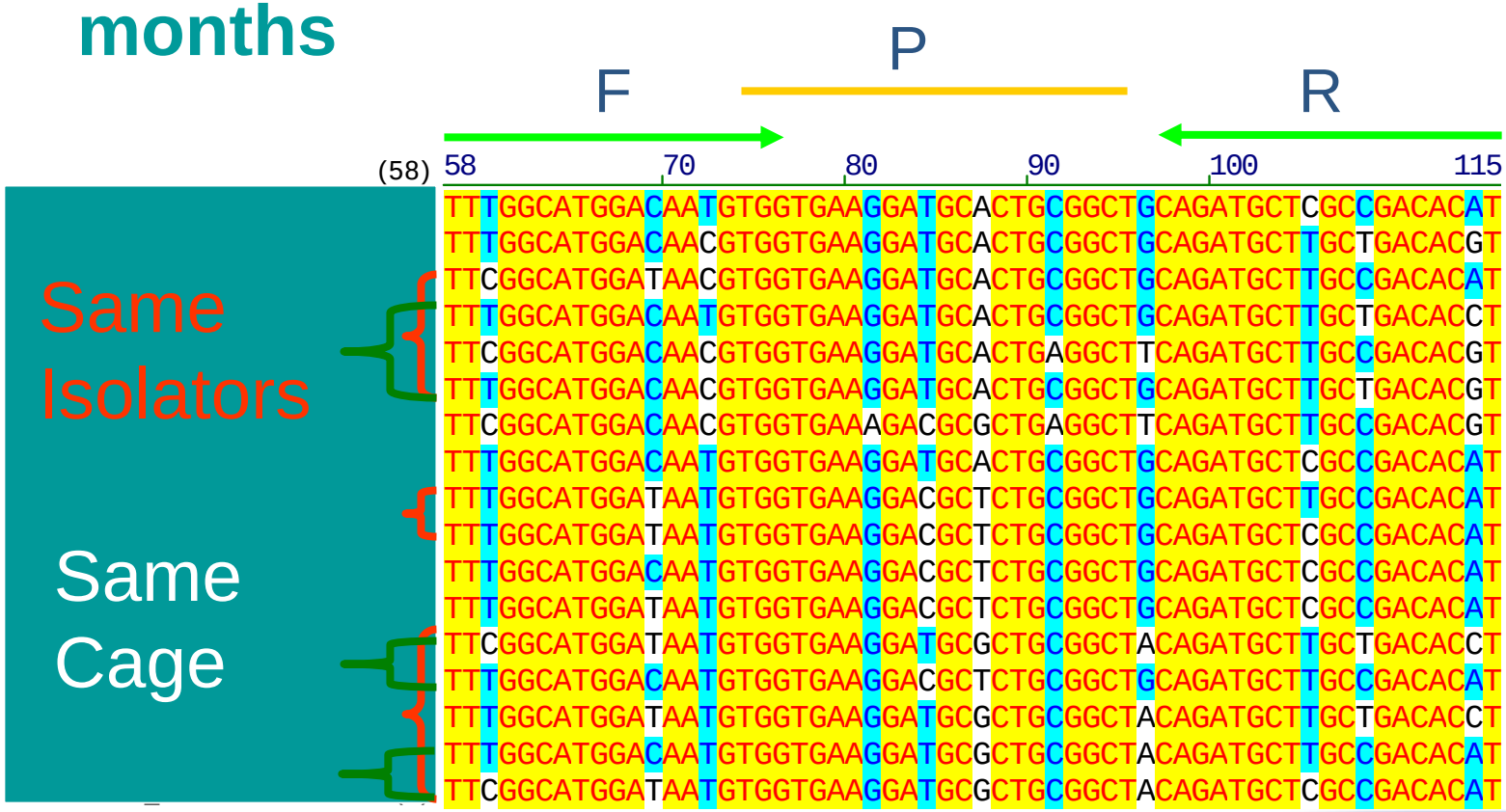
Route	Strain	Titer	
		ELISA	PCR
Gavage	BALB/c	3.2	3.2
	C57BL/6	1.2	1.5
	Δ ID_{50}	**2.0**	**1.7**
IP	BALB/c	2.7	2.7
	C57BL/6	1.7	1.7
	Δ ID_{50}	**1.0**	**1.0**

MNV Surveillance

- Serology
  - ELISA/MFIA: MNV-1 virions, recombinant VP-1
  - IFA: MNV-1, cell culture adapted field isolate
- PCR
  - Nonstructural gene sequence: Polymerase, NTPase
  - ORF1/ORF2
- **Because MNV isolates are so heterogeneous, finding are sufficiently conserved assay target is difficult**

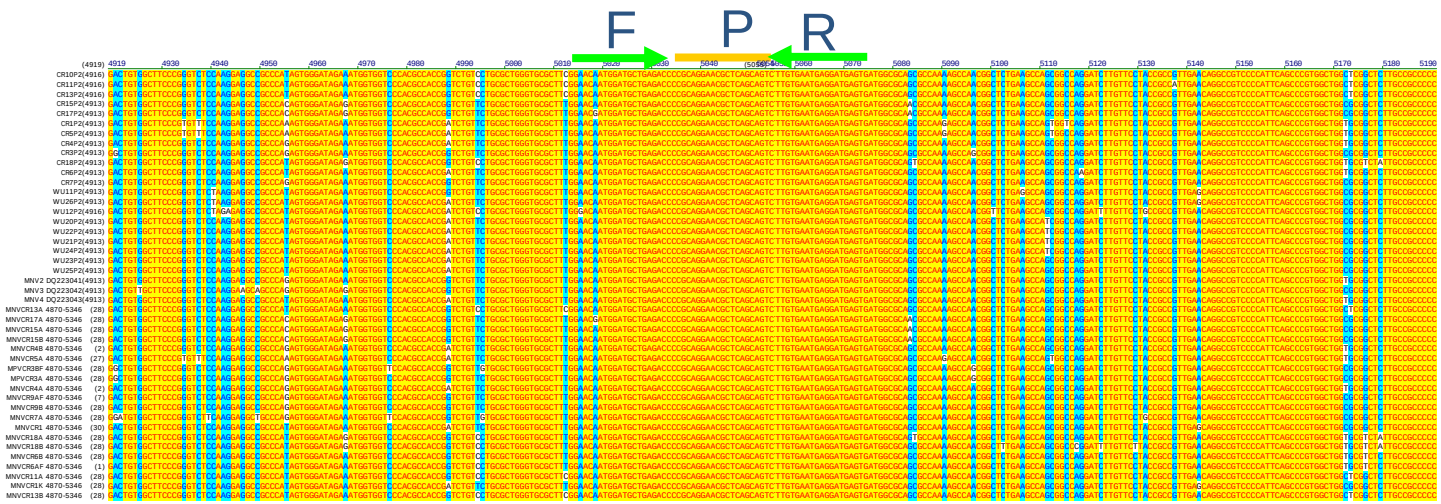
MNV PCR

- "Conserved" nonstructural NTPase gene sequence initially targeted for PCR: **17 variants in 3 months**



MNV PCR: ORF1/ORF2 Junction

- **Current Charles River TaqMan PCR**
  - Use since12/2005
  - Now based on 44 variants



MNV Serology

- rVP1 for ELISA/MFIA
  - Appears to be broadly reactive: Hsu et al. (Comp. Med. 2006) demonstrated cross-reactivity between three field isolates and MNV-1
  - Selected the “middle of the road” MNV variant for Charles River rVP1
- IFA: MNV-1 WU-11field isolate

Serum Antibody Response to 5 MNV Field Isolates: Conventional versus Recombinant Antigen ELISA

MNV Isolate	# Tested	Net Score					
		MNV-CR1a rVP1			Conventional MNV-1		
		Positive		Net Score	Positive		Net Score
		#	%		#	%	
1	4	4	100%	13.6	0	0%	1.3
2	4	4	100%	12.4	0	0%	1.4
3	4	4	100%	13.0	0	0%	0.8
4	4	4	100%	11.2	1	25%	1.2
5	4	4	100%	11.3	2	50%	3.5
MNV-1	4	2	50%	5.6	1	25%	0.8
All	**24**	**22**	**92%**	**11.2**	**4**	**17%**	**1.5**

*Serum was prepared from collected 5 weeks post-inoculation from 4 mice per isolate, including 2 BALB/c and 2 CD-1 mice

Serologic Response of CD-1 and BALB/c Mice to 5 MNV Field Isolates by ELISA, MFIA and IFA

MNV Isolate	CD-1				BALB/c			
	N	Score			N	Score		
		ELISA	MFIA	IFA		ELISA	MFIA	IFA
1	4	21.7	20.7	3.8	4	15.2	17.0	3.5
2	4	21.6	23.2	3.8	4	17.3	17.2	3.0
3	4	22.0	21.8	3.3	3	15.7	15.2	3.0
4	4	21.9	23.2	3.3	4	12.7	13.8	2.0
5	4	21.7	21.8	3.8	4	15.0	13.6	2.5
MNV-1	4	19.8	22.9	3.8	4	13.5	14.8	2.0
All	**24**	**21.4**	**22.3**	**3.6**	**23**	**14.9**	**15.3**	**2.7**

MNV Surveillance: Sentinels go from Sero/PCR-Positive to Negative

- **Initial results false-positive**
  - Sampling Error
  - Laboratory Error
- **Results are false-negative**
  - Sampling Error
  - Laboratory Error
  - Loss of Assay Sensitivity

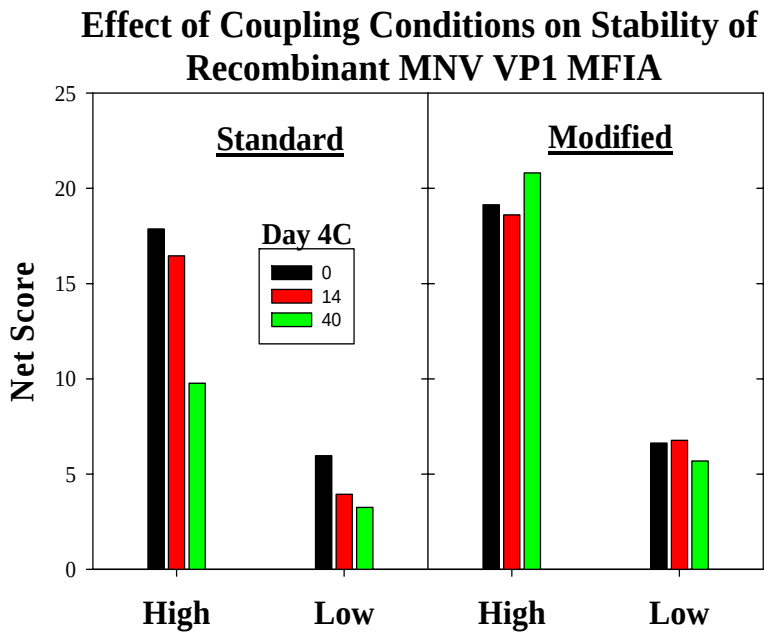
MNV Surveillance: Sentinels Serology/PCR-Positive to Negative

- Loss of assay sensitivity due to antigen instability

Effect of MNV-Bead Lot on MFIA Scores for Temporally Sampled Sentinels*

Sampling Date	Antigen Bead Lot	MFIA Score	
		S1	S2
17-Nov-06	1	3	19
9-Jan-07	1	3	1
	2	11	7

*S1 and S2



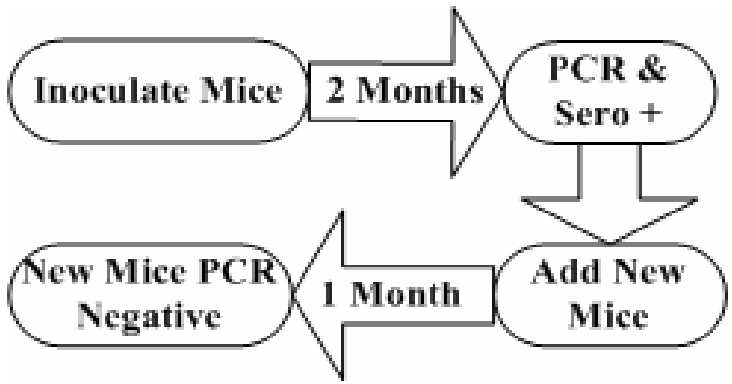
MNV Surveillance: Sentinels Serology/PCR-Positive to Negative

- **Not exposed to infectious dose of virus**
  - Soiled bedding not transferred
  - **Principal mice no longer contagious even though fecal PCR +**

Disparity Between Sentinel Groups

	Sentinels			Principals	
Month	Group	MFIA	PCR	MFIA	PCR
0	1	+	+	+	+
3	2	-	-		+

Lack of Transmission from Experimentally Infected Mice



Prevention and Detection of Adventitious MPV and MNV Infections: **Summary**

- **Adventitious infections of SPF lab rodent models, even if asymptomatic, can alter and confound research findings**
- Advances in biosecurity practices have dramatically reduced the prevalence of once common adventitious agents
- MPV and MNV, however, continue to be prevalent because:
  - They were recently discovered
  - Produce asymptomatic, persistent infections of mice
  - Are resistant to disinfection because of their physicochemical characteristics

Prevention and Detection of Adventitious MPV and MNV Infections: **Summary**

- As infections of mice with these agents are asymptomatic, monitoring requires **laboratory testing by Serology and PCR**
- Colony test results may be inconsistent due to:
  - **Sampling Error:** Inadequate exposure of sentinels
  - **Laboratory Error:** Assay- or operator-related
  - **Pathobiology of Virus**
    - Convalescent mice may not be contagious
    - Differences in susceptibility to infection between sentinel and principal mice