

**"Workshop di Microscopia Elettronica
nelle Malattie Infettive - Diagnostica e Ricerca"
IZS del Lazio e Toscana - 08 Novembre 2013– Roma (Italy)**

USE AND ROLE OF ELECTRON MICROSCOPY FOR THE DIAGNOSIS OF ANIMAL VIRUSES IN VETERINARY PRACTICE

**Antonio Lavazza (IZSLER)
Giusy Cardeti (IZSLT)**

Aim of the presentation



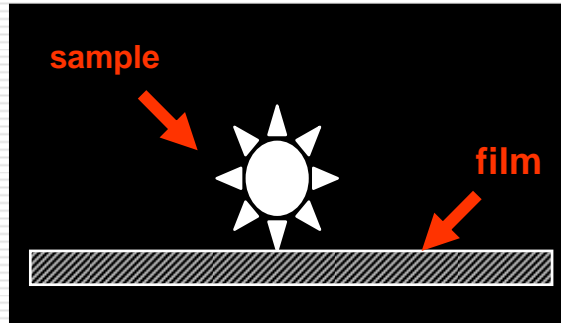
To give, through some examples coming from our work experience,
an overview of the use of negative staining EM methods for the detection and identification of viral particles in animal samples

Summary



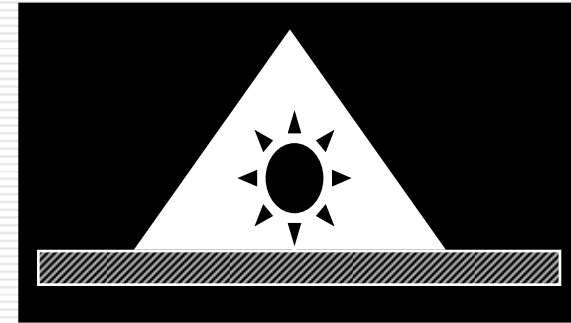
- × EM use in vet virology
- × Enteric viruses
- × Vector-borne/Insect viruses
- × Viruses in minor species and wild animals
- × Fish viruses
- × Honeybee viruses
- × Poxviruses
- × IEM with convalescent sera
- × Immunogold
- × Conclusions

Positive and negative staining



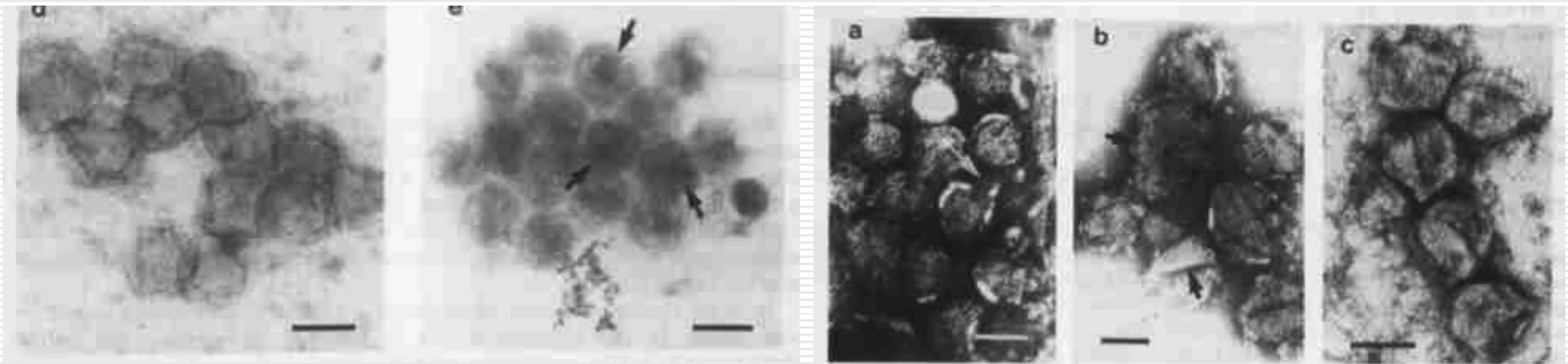
POSITIVE

Uranyl acetate
Lead citrate
NaPT pH acid (4.5)



NEGATIVE

NaPT pH neuter (7.2)
Ammonium molybdate



Negative staining EM (nsEM) methods

Advantages:

- easy and quick to perform
- good conservation, contrast and resolution
- need of low amount of sample and staining

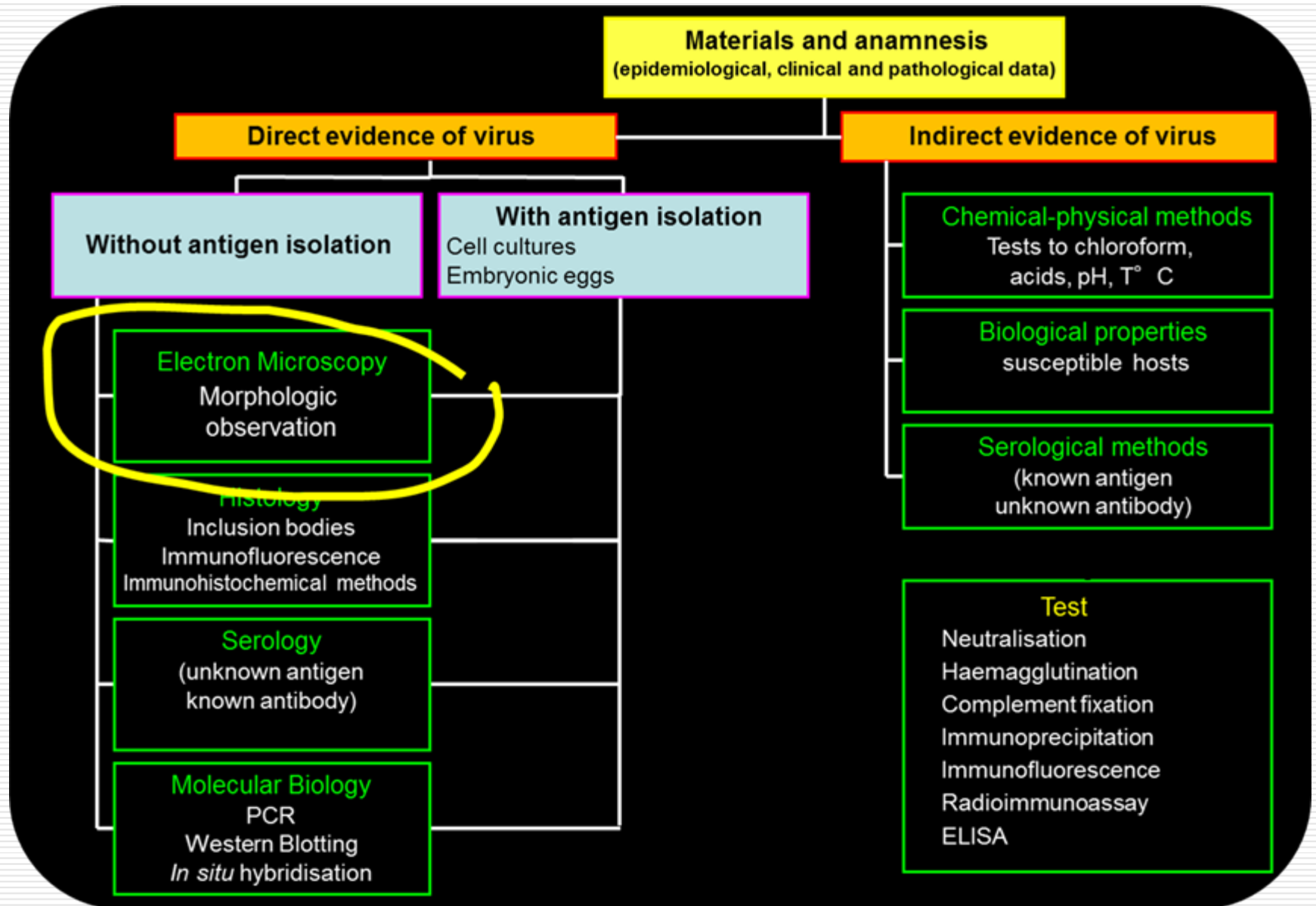
Disadvantages:

- Limited to the study and detection of particles in water suspension
- Need of quite elevated concentration of particles (at least 10^3)



**PARTICULARLY USEFUL
FOR DIAGNOSIS OF VIRUSES**

Work flow in virological diagnosis



nsEM peculiarities

Positive

- Results achieved in short times
- Permit to observe viruses that could not be isolated or identified by serological methods
- Permit to detect multiple viral infection (viruses in association)
- Permit to observe virions no more able to replicate (immuno-aggregates)

Negative

- Limited to few labs
- Sensitivity depends from the preparation method applied
- Not useful for those viruses which do not have specific morphological characteristics
- Identification of viruses at "family level" (by-passed using IEM)

Morphological identification of viruses

NECESSARY:

to maintain the structural characteristics and the architecture of the examined object

BASED ON:

- shape, size, symmetry
- number and size of capsomers
- presence, shape and size of peripheral projections (spikes)
- type of organization of the nucleic acid



VIRAL FAMILY

+ IEM



GENUS, SPECIES, SEROTYPE

Negative staining EM for viral diagnosis

Quite good level of sensitivity
(depending on the method used)



Method	Threshold level	Time
Drop method	10^6 - 10^8 particles/ml	Few min.
Airfuge method	10^4 - 10^6 particles/ml	~ 3 hours
IEM-Airfuge	10^3 - 10^4 particles/ml	~ 4 hours
IEM-Gold	10^3 - 10^4 particles/ml	~ 5 hours

The "drop" method

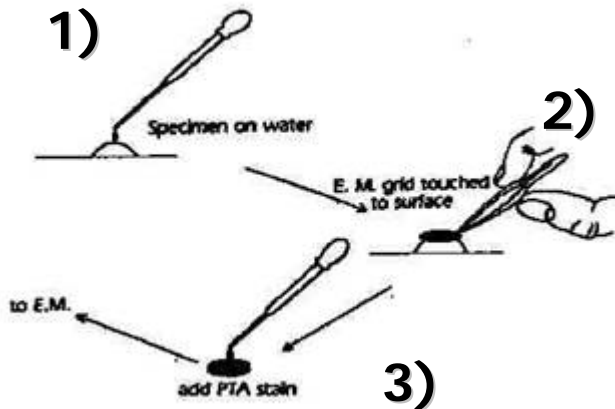
Cell culture supernatant and/or lysate

Purified virus, suspensions or fractions

Egg-embryo (eg. allantoic fluid)

Crusts, papule, vesicles, nodular lesions

- 1) One drop of virus suspension (if needed, after sonication or fixation in 0.2% glutaraldehyde)
- 2) one grid is put on the drop to float on its Formvar face
- 3) after 10 min contact, 2% NaPT stain is added (1.5 min)



Examination at 80kV - 16000 - 25000 x

The "Airfuge" method

1) DILUTION of FAECAL SAMPLES

1:5 w/v in bi-distilled water

2) LOW SPEED CENTRIFUGATIONS

6000 and 10000 rpm for 30 min

3) IEM STEP

incubation with serum at 37°C for 1 hr

4) ULTRACENTRIFUGATION AIRFUGE BECKMAN

21 psi for 15 min

5) NEGATIVE STAINING

2% NaPt, pH 6.8 for 1.5 min

6) OBSERVATION

TEM - 80 kV at 19000-34000x



Elements for Airfuge ultracentrifugation

Supernatant from
2nd centrifugation

A-100 rotor
microtube

adapter



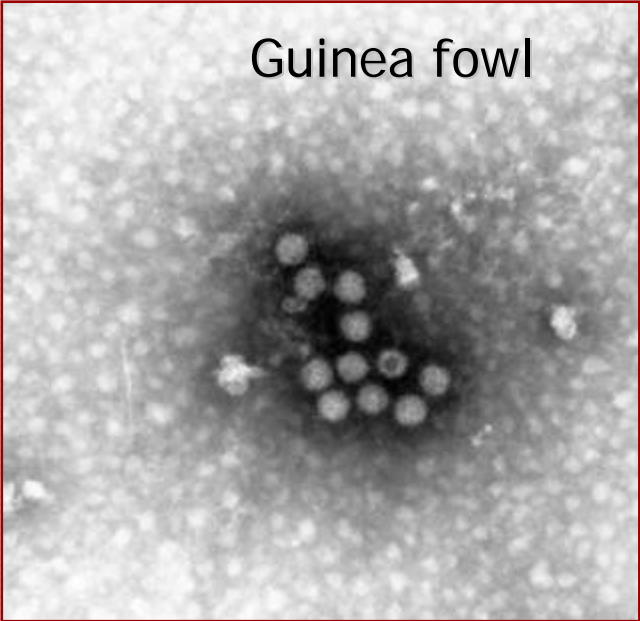
Diagnosis of Enteric viruses

- ✓ often not cultivable virus
- ✓ multiple viral infections

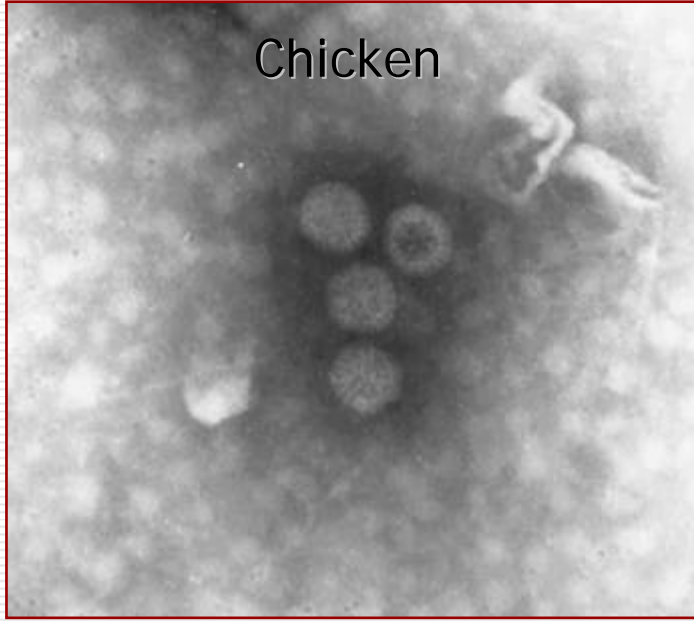


Rotavirus in poultry

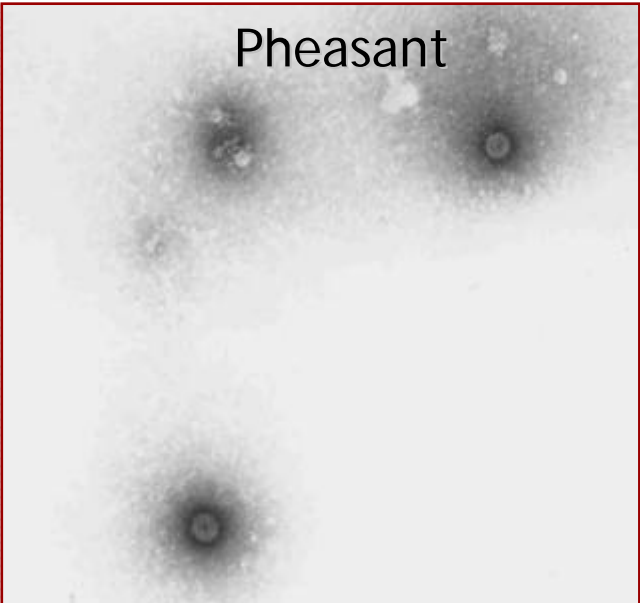
Guinea fowl



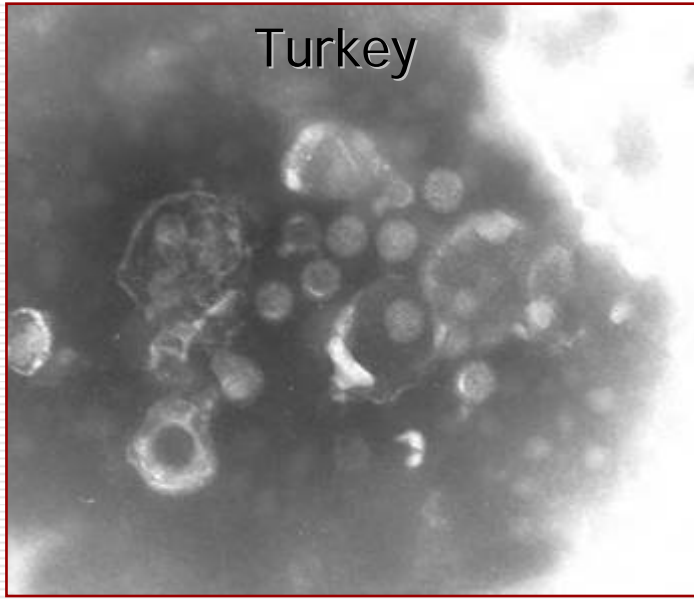
Chicken



Pheasant

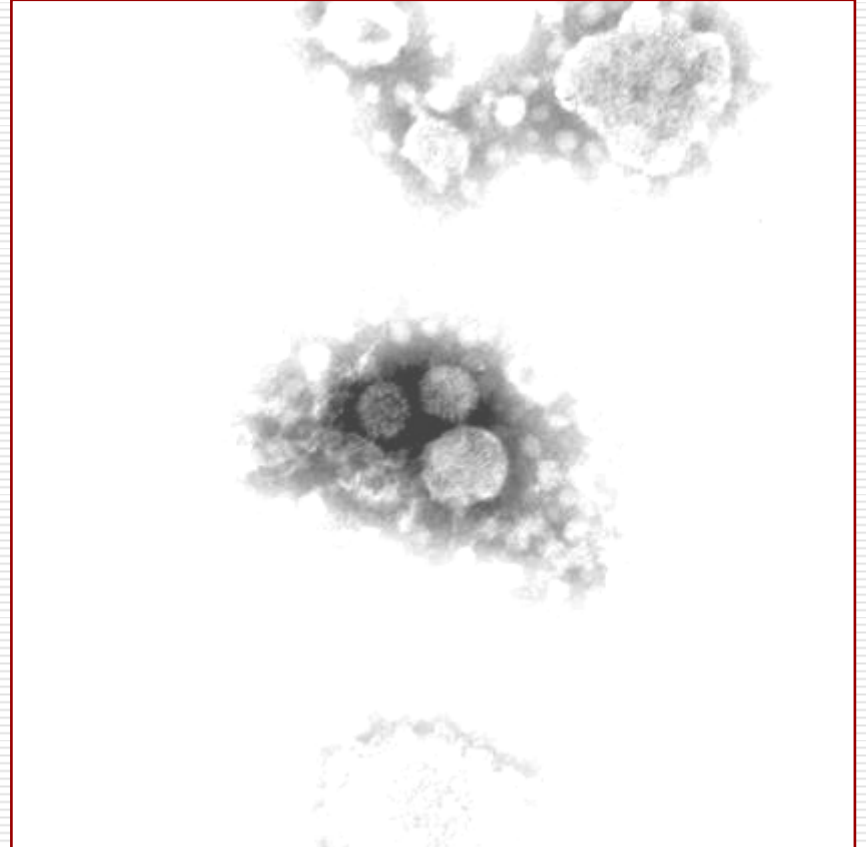
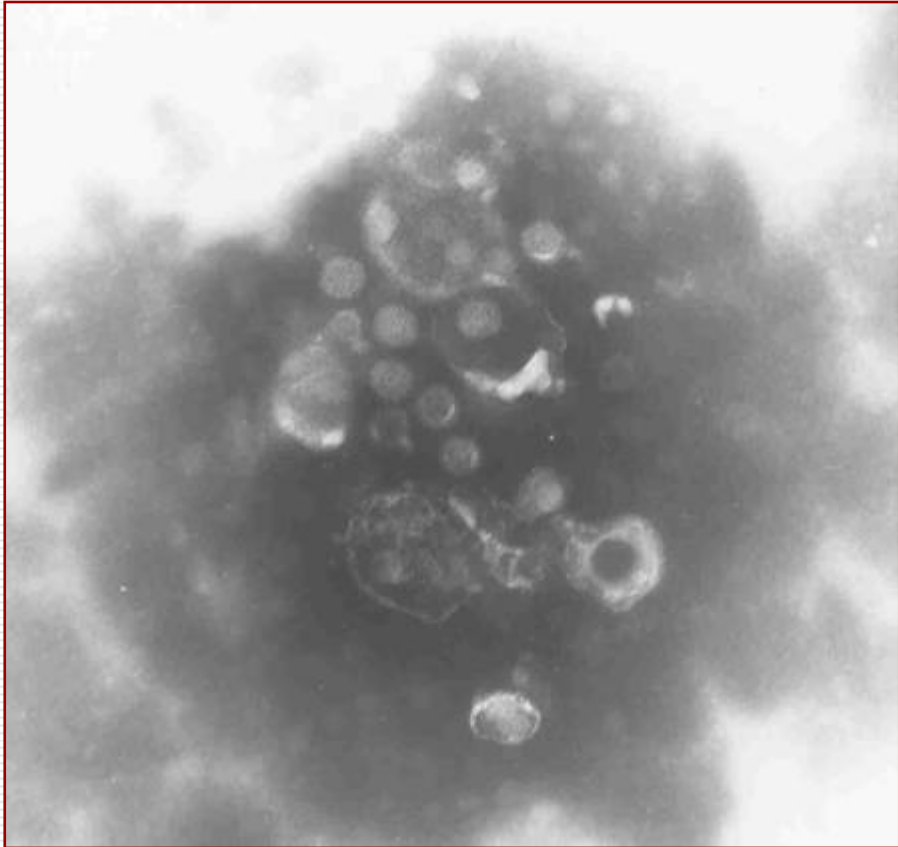


Turkey



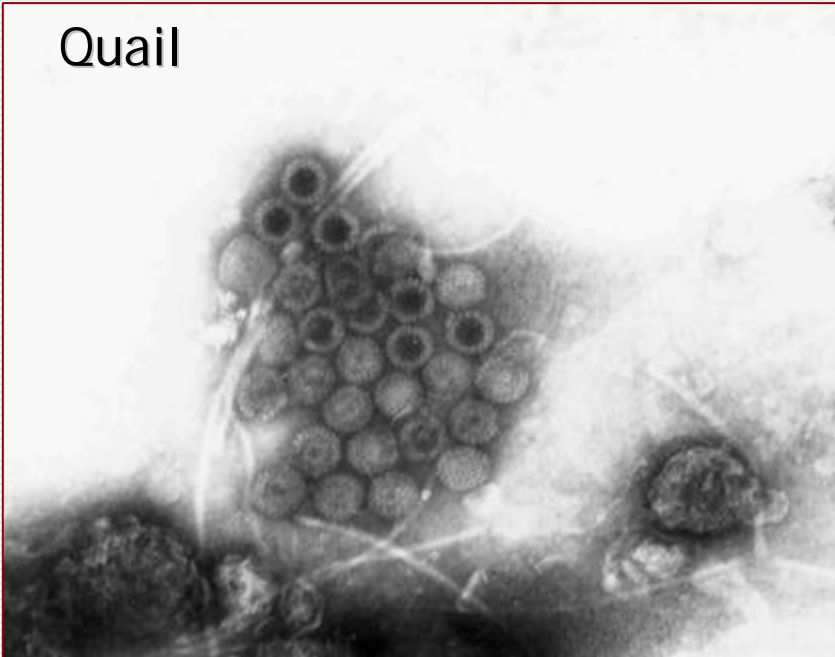
Both Group A
(ELISA pos)
and non-A
(Elisa neg)
Rotavirus

Rotavirus in canary (left) and pigeon (right)

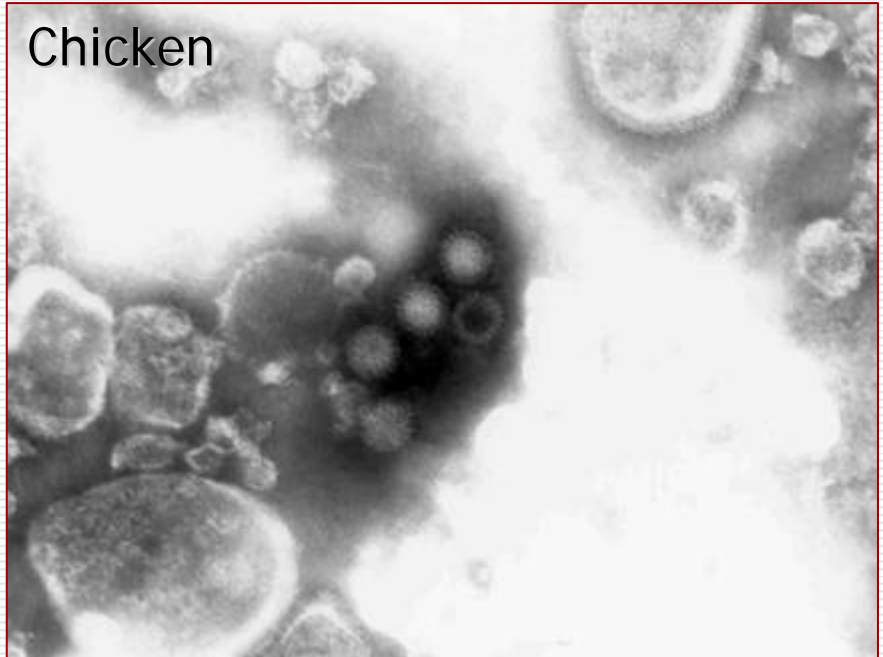


Reovirus in poultry

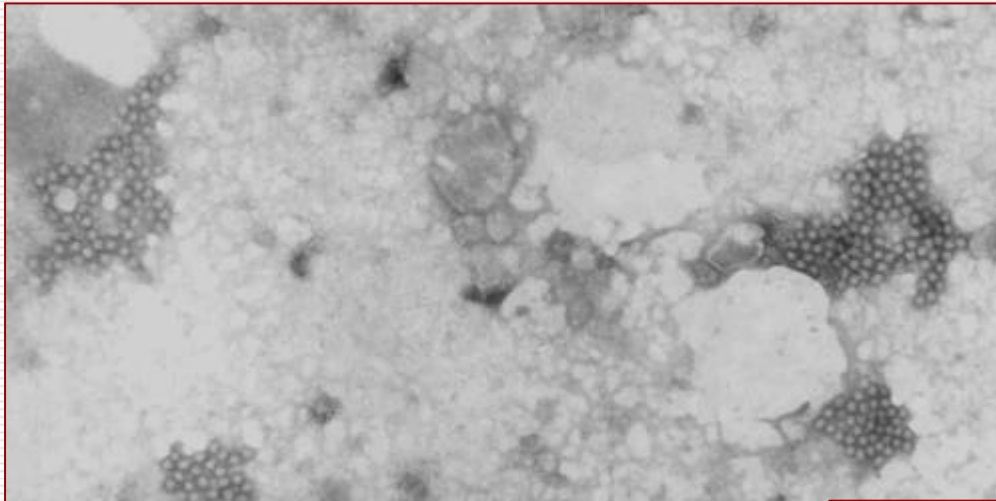
Quail



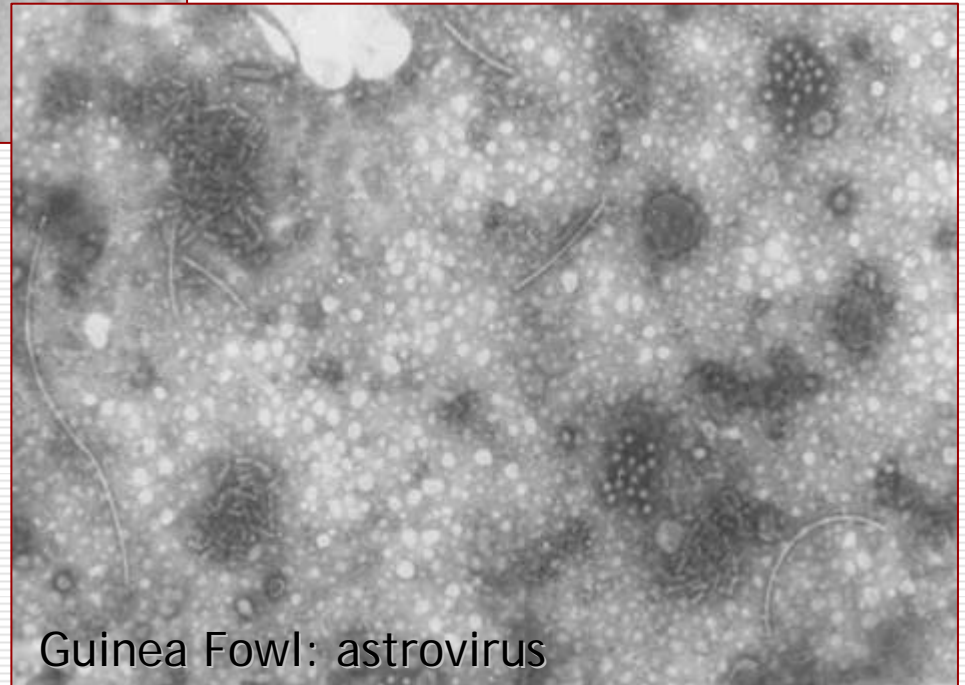
Chicken



Astrovirus (enterovirus-like) in poultry

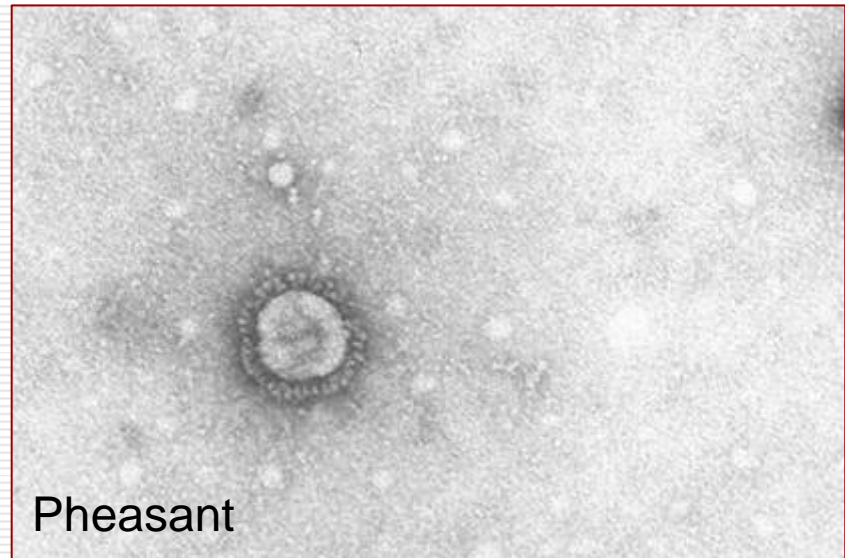
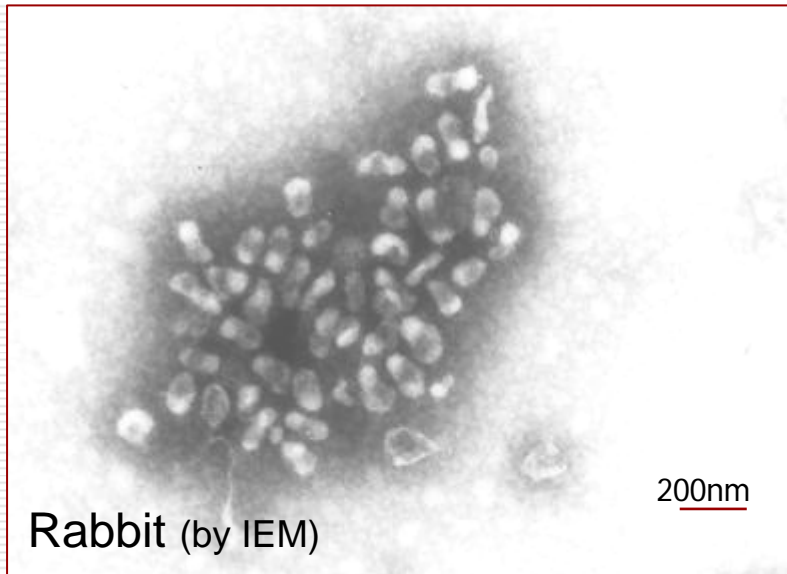
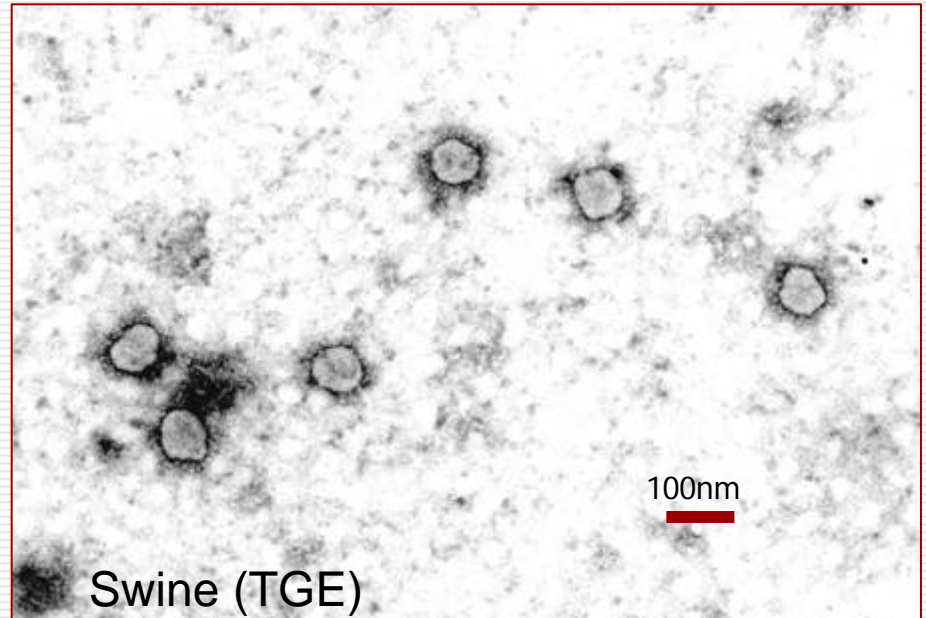
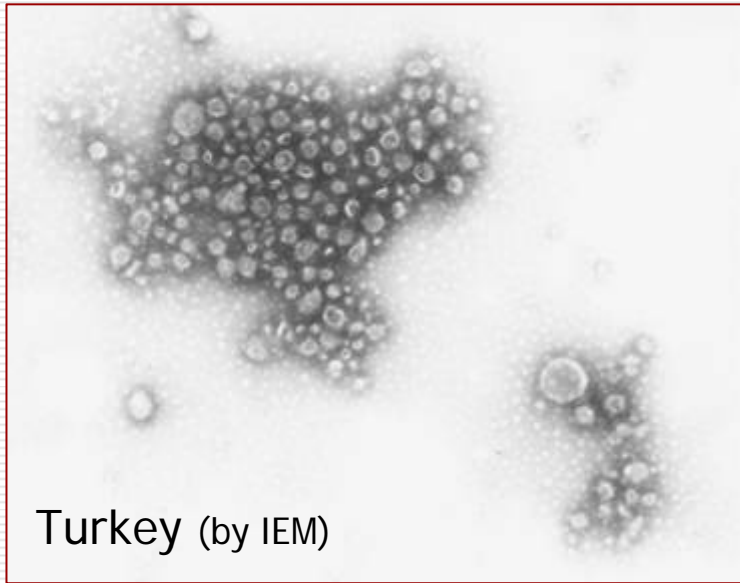


Chicken: SRV (enterovirus-like)



Guinea Fowl: astrovirus

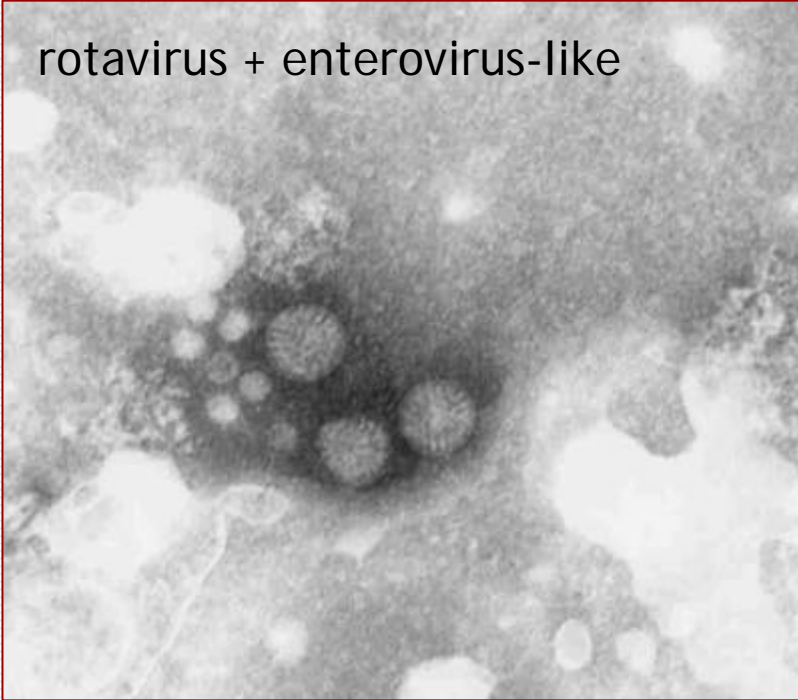
Coronavirus



Viral association in poultry gut contents

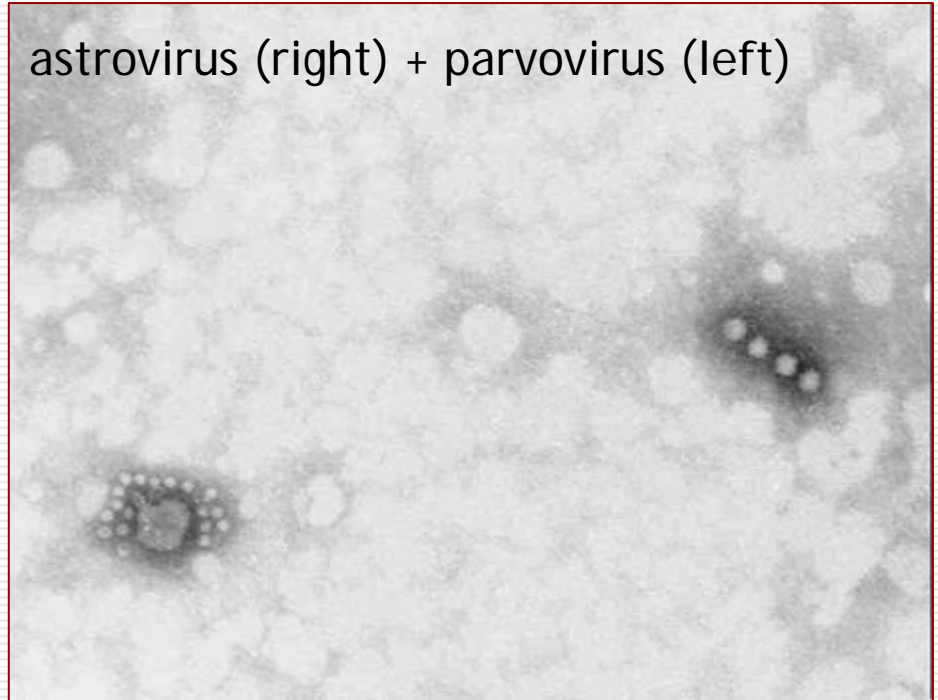
Chicken

rotavirus + enterovirus-like



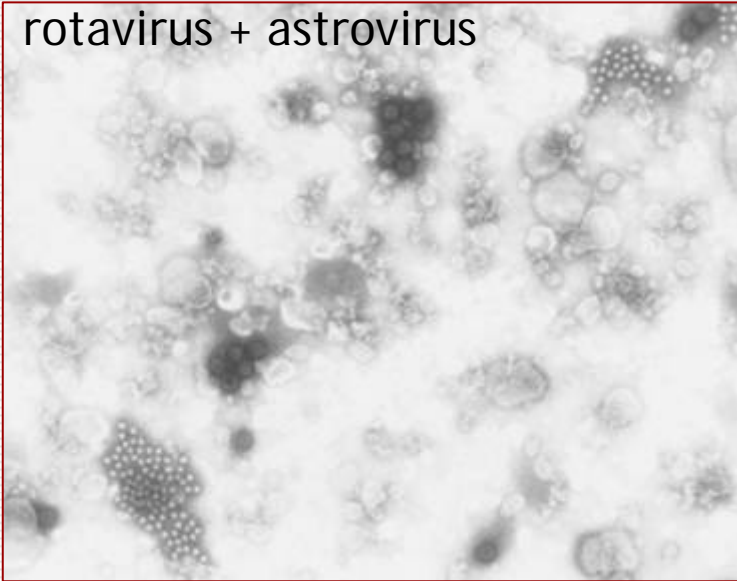
Guinea fowl

astrovirus (right) + parvovirus (left)

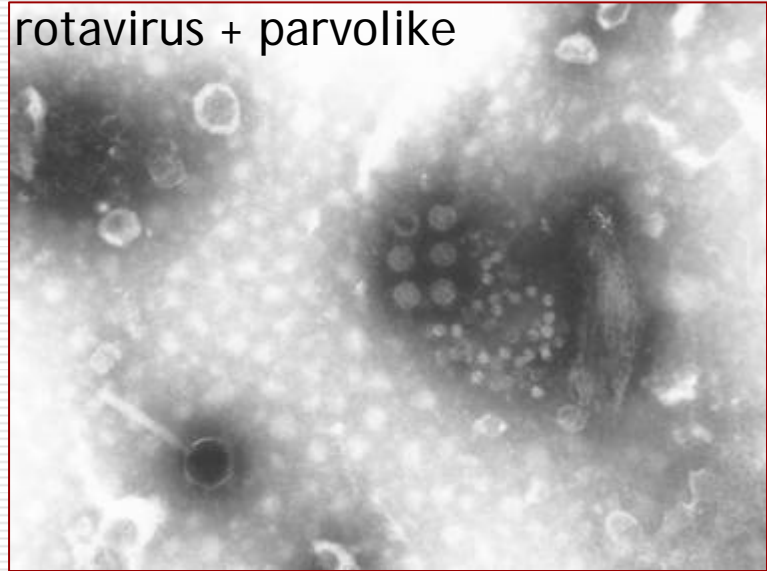


Viral association in turkey gut contents

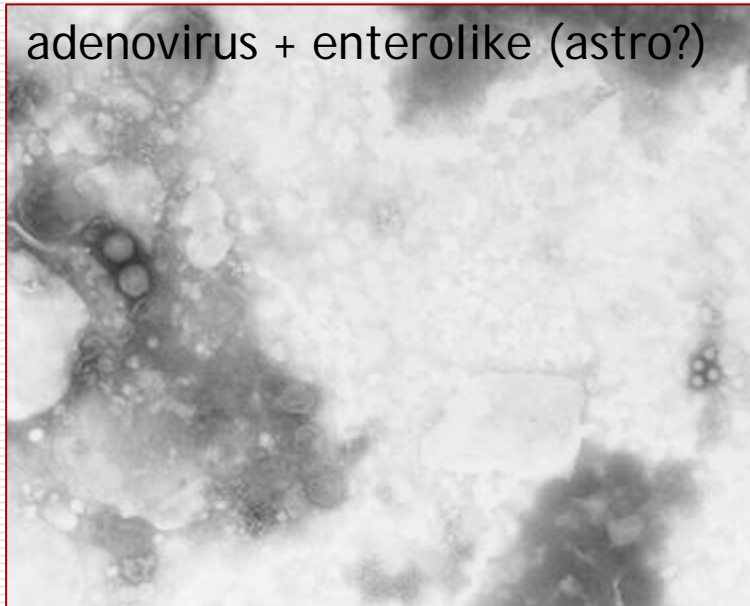
rotavirus + astrovirus



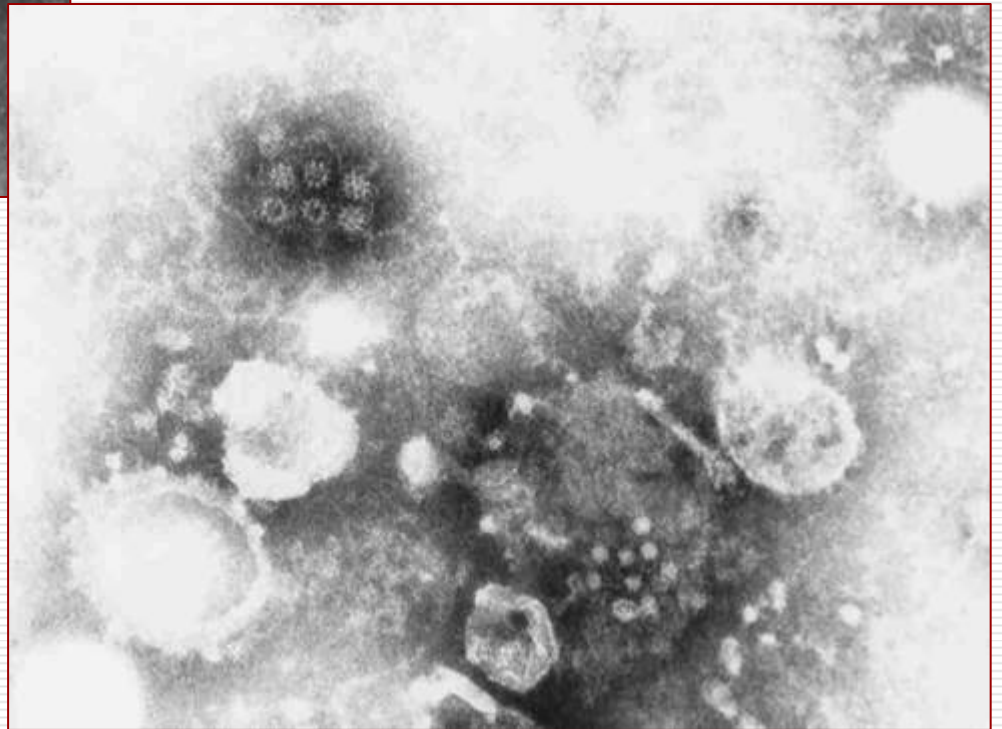
rotavirus + parvolike



adenovirus + enterolike (astro?)



Calicivirus and Parvovirus in pheasant



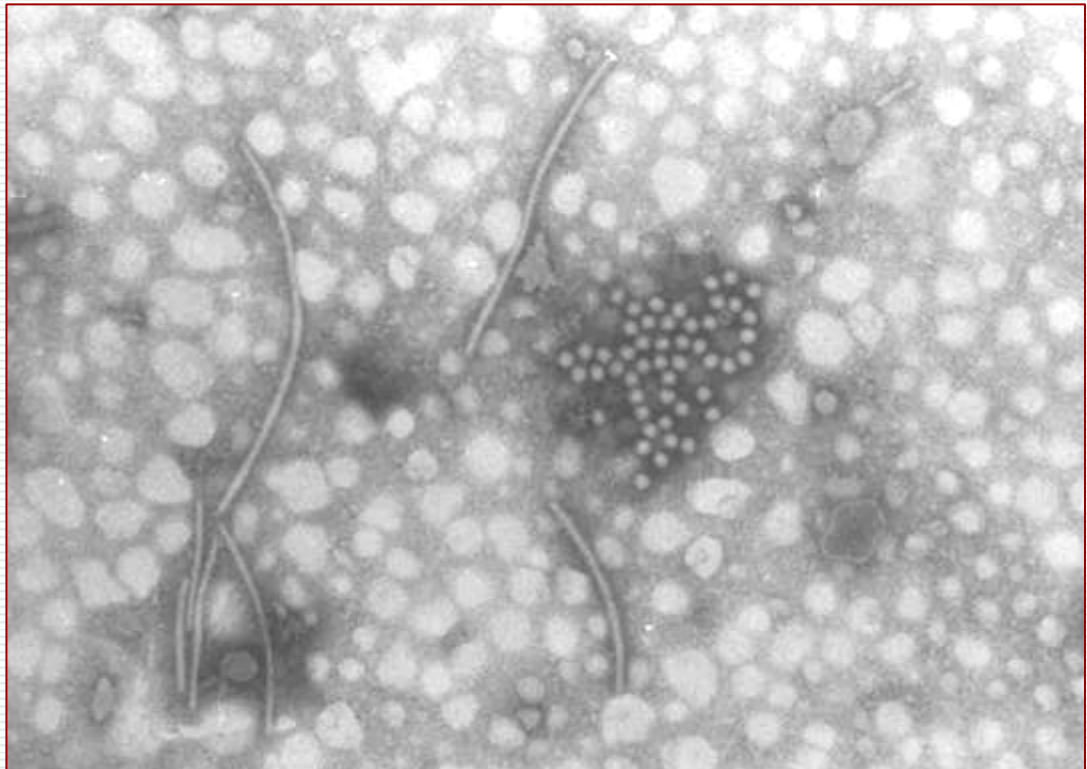
Circovirus (CIAV) + Birnavirus (IBDV) in chicken



Enterovirus-like in pigs

In diarrheic pigs, many SRV (*enterovirus-like*, *calicivirus-like* and *astrovirus-like*) are sporadically observed

They need to be further characterized (*norovirus*, *sapovirus*, *anellovirus*, *bocavirus*, *hepevirus*.....)



ORIGINAL ARTICLE

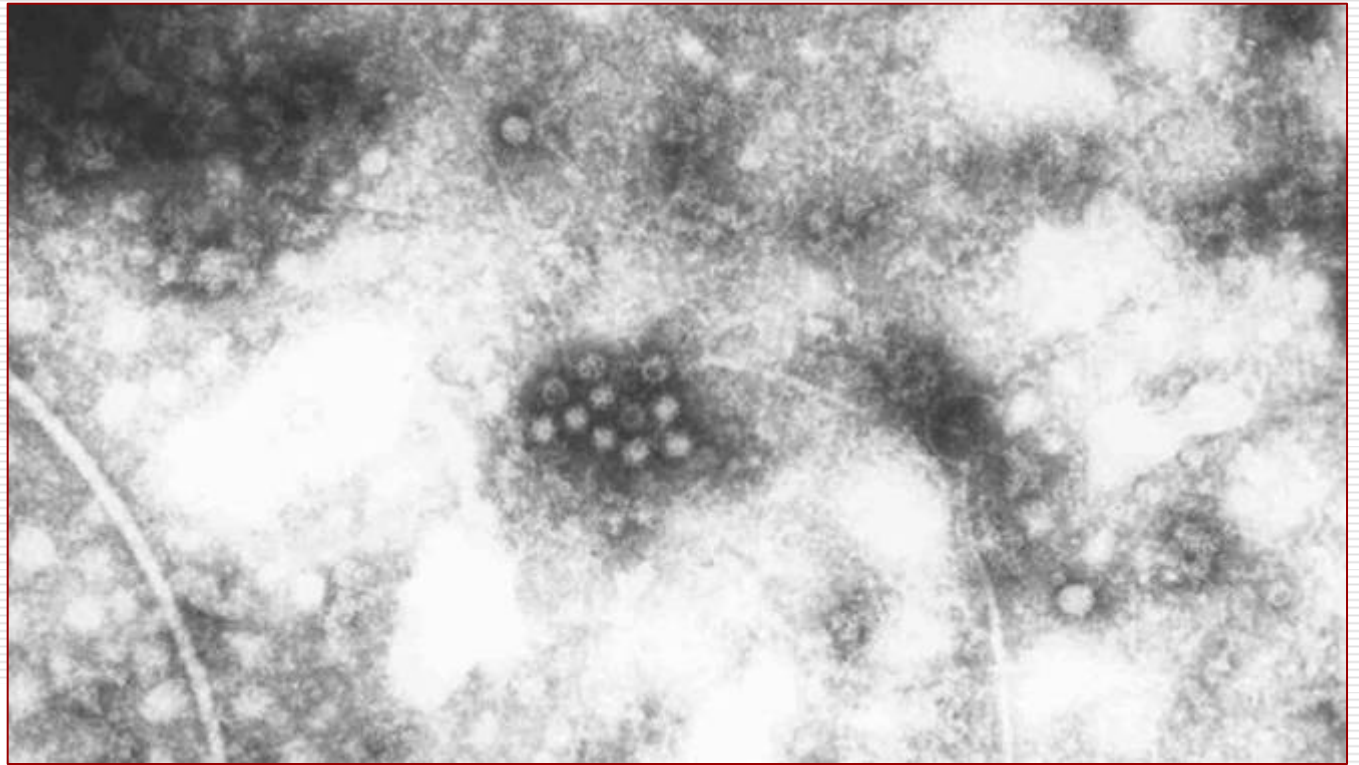
Molecular Characterization and Phylogenetic Analysis of VP1 of Porcine Enteric Picornaviruses Isolates in Italy

E. Sozzi, I. Barbieri, A. Lavazza, D. Lelli, A. Moreno, E. Canelli, M. Bugnetti and P. Cordioli

Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna 'B. Ubertini', Brescia, Italy

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Calicivirus

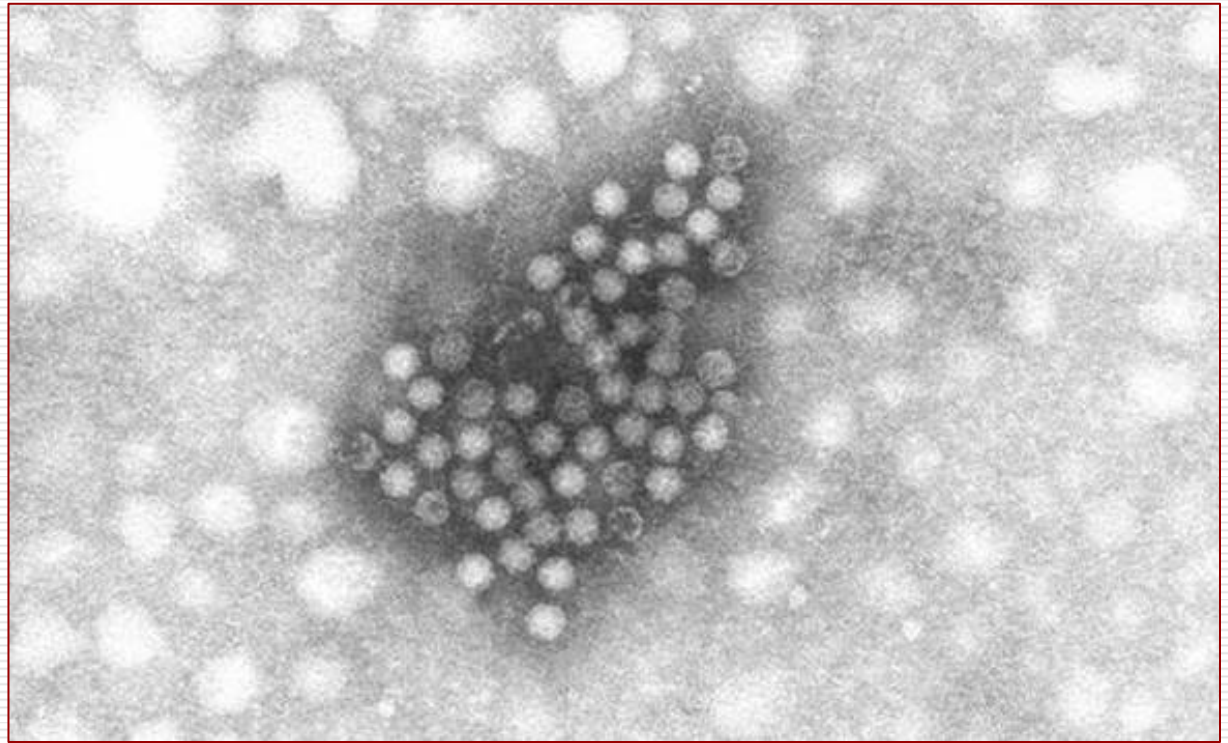


Virus Genes
DOI 10.1007/s11262-008-0198-0

Genetic heterogeneity of porcine enteric caliciviruses identified from diarrhoeic piglets

V. Martella · K. Bányai · E. Lorusso · A. L. Bellacicco · N. Decaro ·
V. Mari · L. Saif · V. Costantini · S. De Grazia · G. Pezzotti · A. Lavazza ·
C. Buonavoglia

Calicivirus-like - Sapovirus -



JOURNAL OF CLINICAL MICROBIOLOGY, June 2008, p. 1907–1913
0095-1137/08/\$08.00+0 doi:10.1128/JCM.00341-08
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Vol. 46, No. 6

Identification of a Porcine Calicivirus Related Genetically to Human Sapoviruses[▽]

V. Martella,^{1*} E. Lorusso,¹ K. Banyai,² N. Decaro,¹ M. Corrente,¹ G. Elia,¹ A. Cavalli,¹ A. Radogna,¹
V. Costantini,³ L. J. Saif,³ A. Lavazza,⁴ L. Di Trani,⁵ and C. Buonavoglia¹

Department of Public Health and Zootechnic, University of Bari, Valenzano, Bari, Italy¹; Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary²; Food Animal Health Research Program, Ohio Agricultural Research and Development Center, Department of Veterinary Preventive Medicine, The Ohio State University, Wooster, Ohio 44691³; Istituto Zooprofilattico Sperimentale di Lombardia/Emilia Romagna, Brescia, Italy⁴; and Istituto Superiore di Sanità, Dipartimento di Sanità Alimentare e Animale, Rome, Italy⁵

Pig and Bovine Torovirus

JOURNAL OF VIROLOGY, Sept. 2003, p. 9567-9577
0022-538X/03/\$08.00+0 DOI: 10.1128/JVI.77.17.9567-9577.2003
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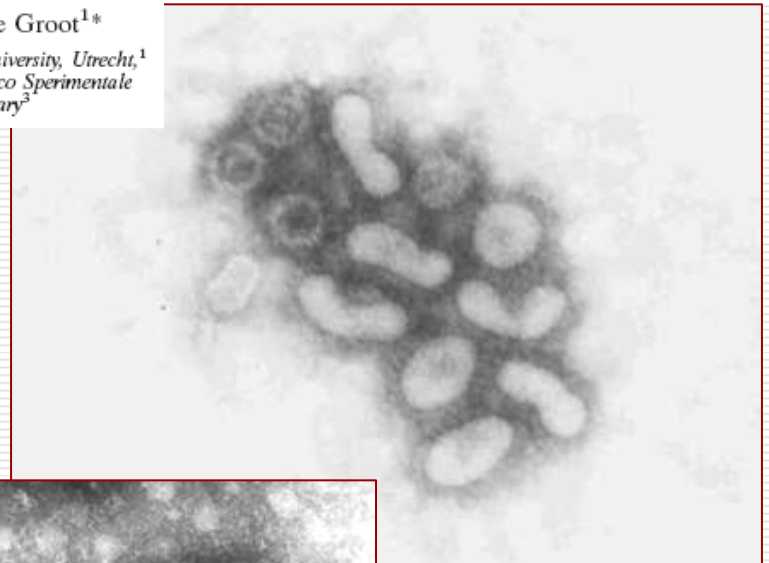
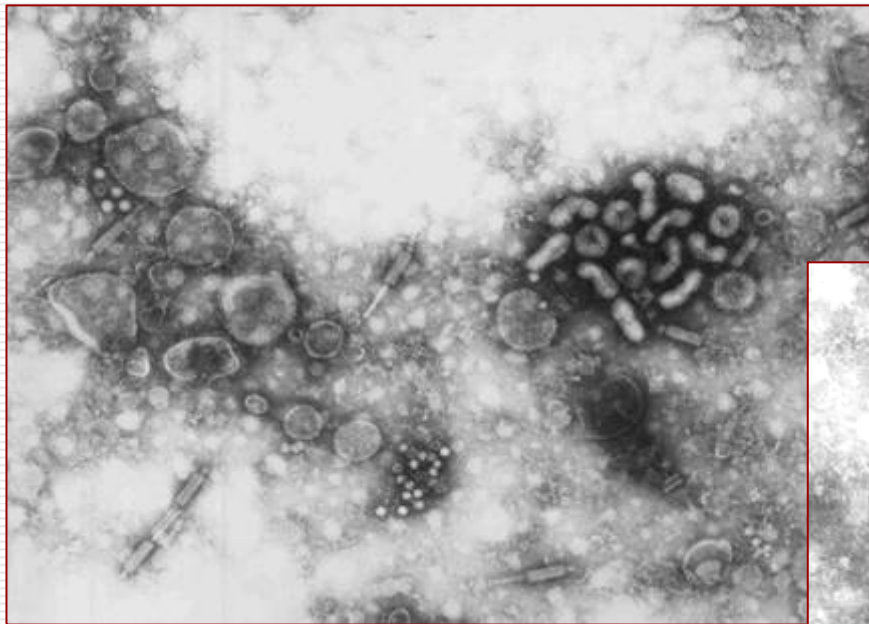
Vol. 77, No. 17

Phylogenetic and Evolutionary Relationships among Torovirus Field Variants: Evidence for Multiple Intertypic Recombination Events

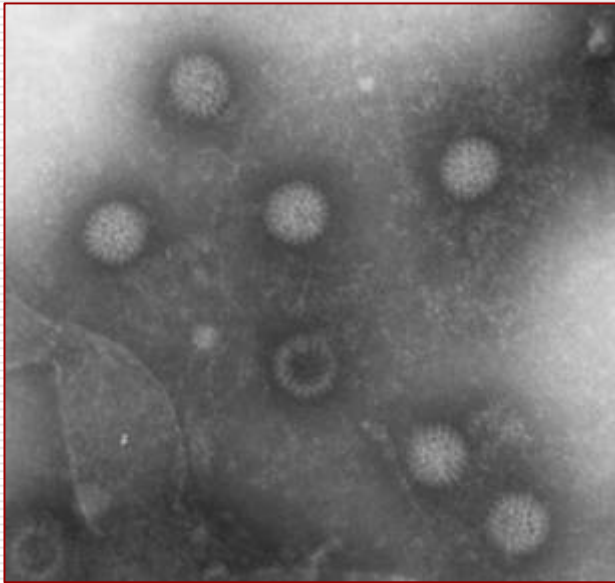
S. L. Smits,¹ A. Lavazza,² K. Matiz,³ M. C. Horzinek,¹ M. P. Koopmans,⁴ and R. J. de Groot^{1*}

Virology Division, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht,¹ and National Institute of Public Health and the Environment, Bilthoven,⁴ The Netherlands; Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Brescia, Italy²; and Veterinary Institute of Debrecen, Debrecen, Hungary³

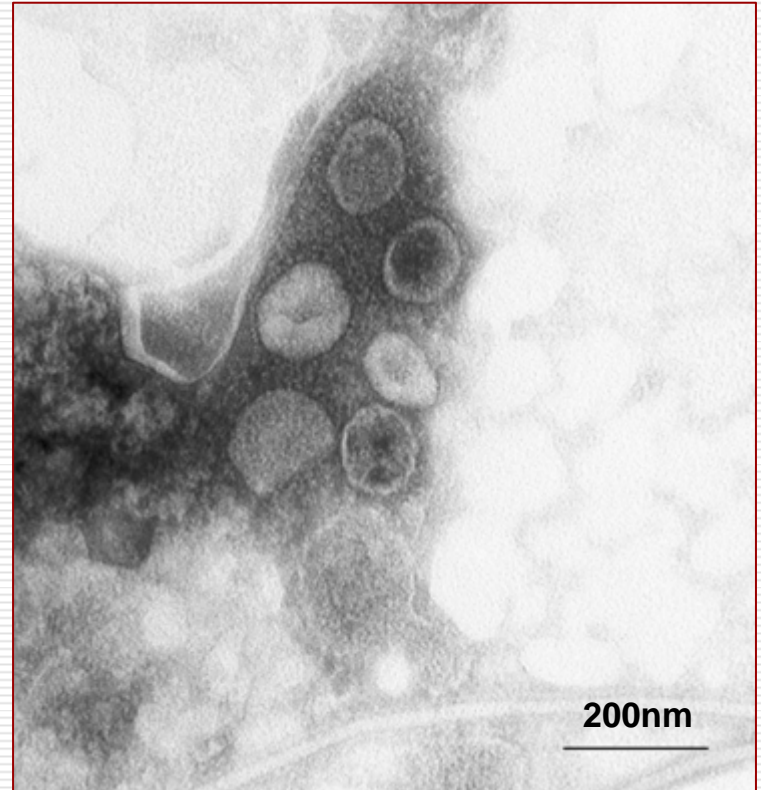
The pathological significance of these agents has to be fully ascertained



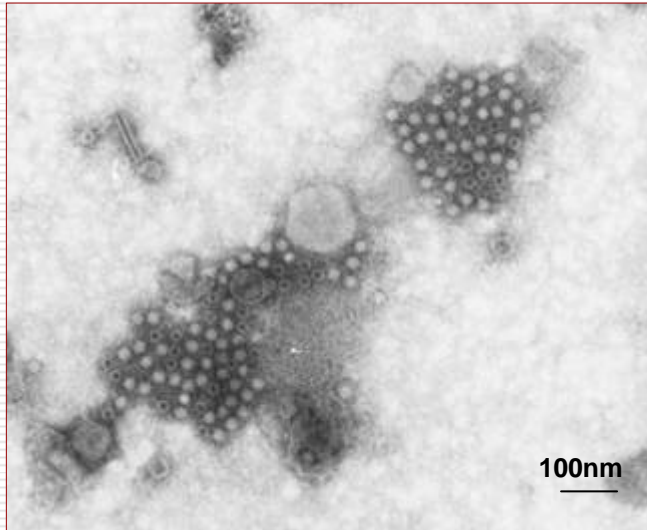
Enteric viruses in rabbits



Rotavirus = the most important viral agent causing enteritis in rabbits

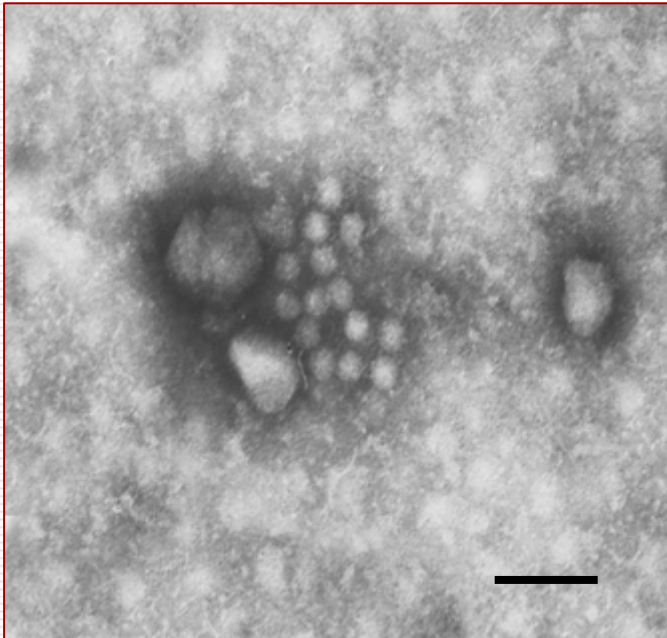
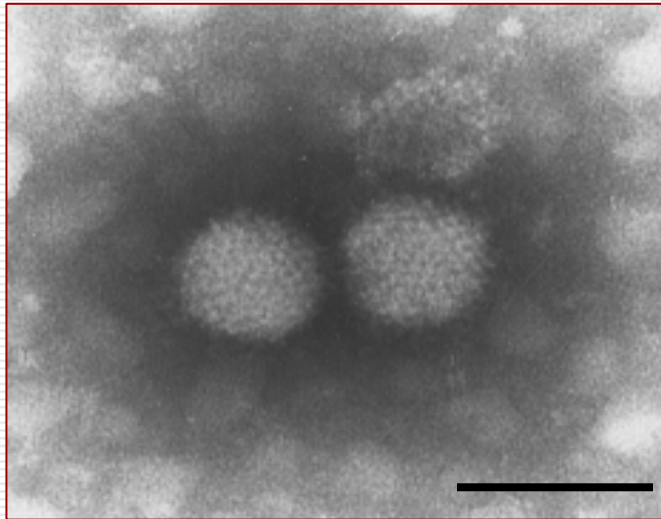


Parvovirus =
It has been considered a common pathogen with very low or null virulence

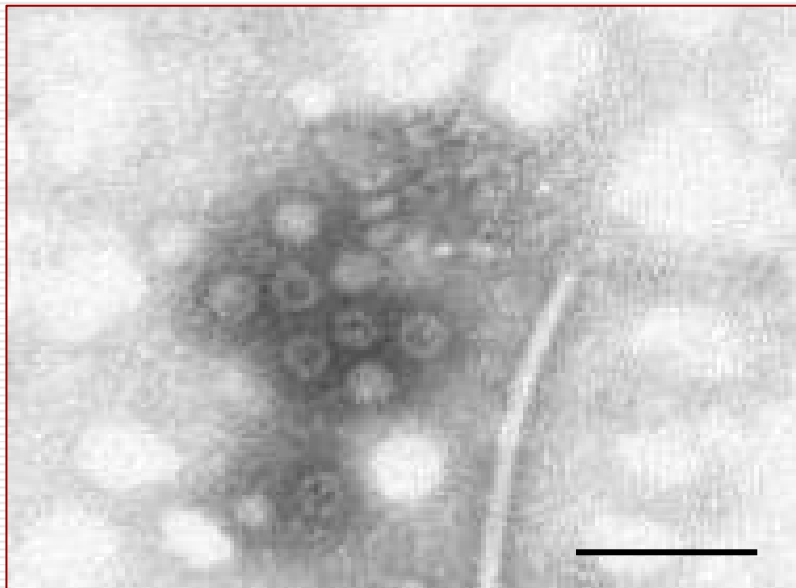


Coronavirus = Described as agent of systemic and enteric disease. Pathogenic role not well cleared

Adenovirus = Very rarely identified, few reports. Post-weaning diarrhea



Enterovirus-like = Recently reported, few reports. Poor characterization. Pathogenic role not yet defined



Calicivirus (vesivirus) = Isolated from rabbits with diarrhea. Correlated to vesicular exanthema virus and to cat and dog calicivirus

Diagnosis of vector-borne/insect viruses

- ✕ Directly from pathological materials and individuals (insects)
- ✕ From cell culture supernatants / lysates

Orbivirus (family Reoviridae)



Alphavirus (family Togaviridae)

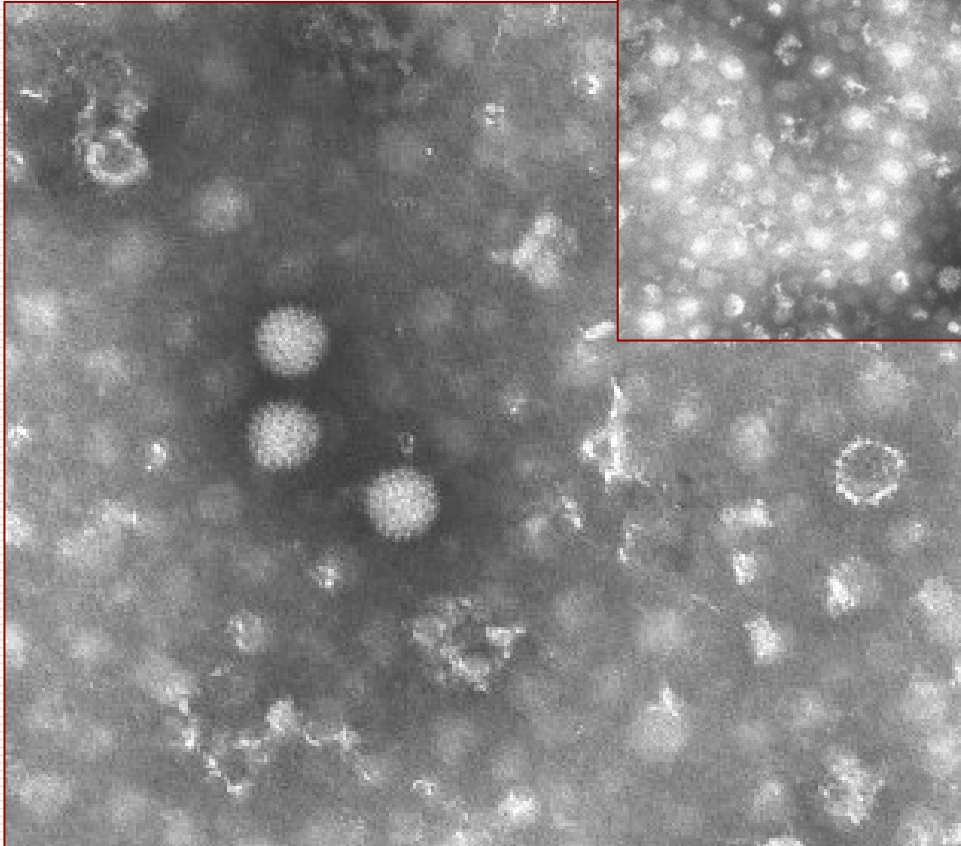
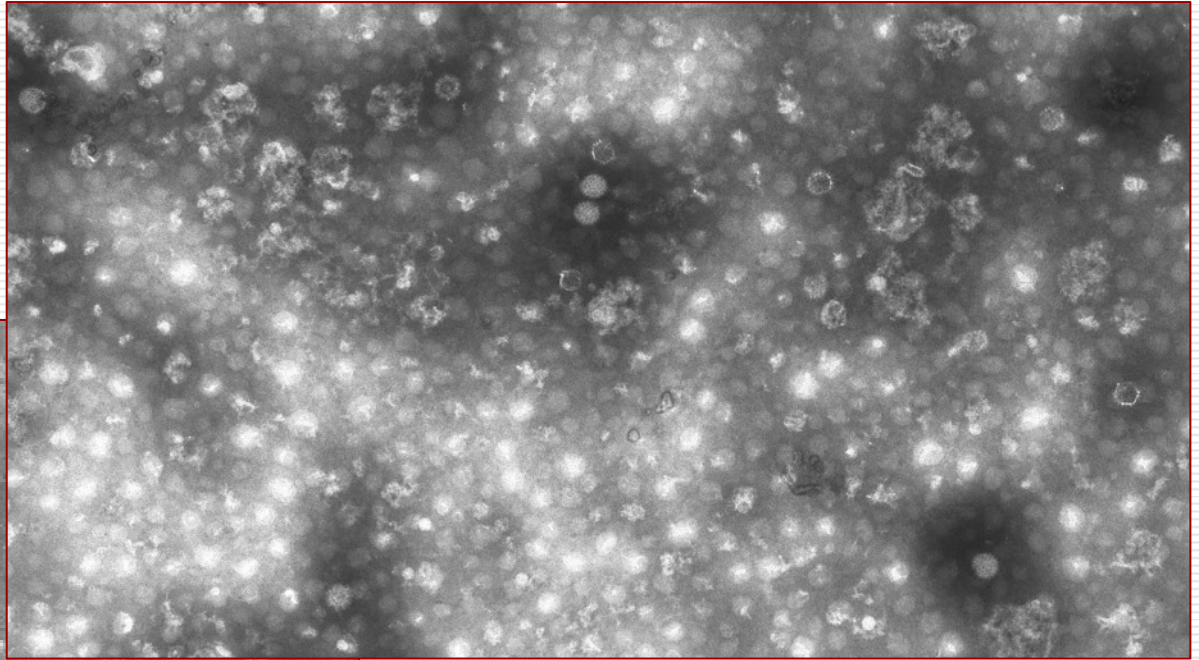


Flavivirus (family Flaviviridae)

Orthobunyavirus (family Bunyaviridae)

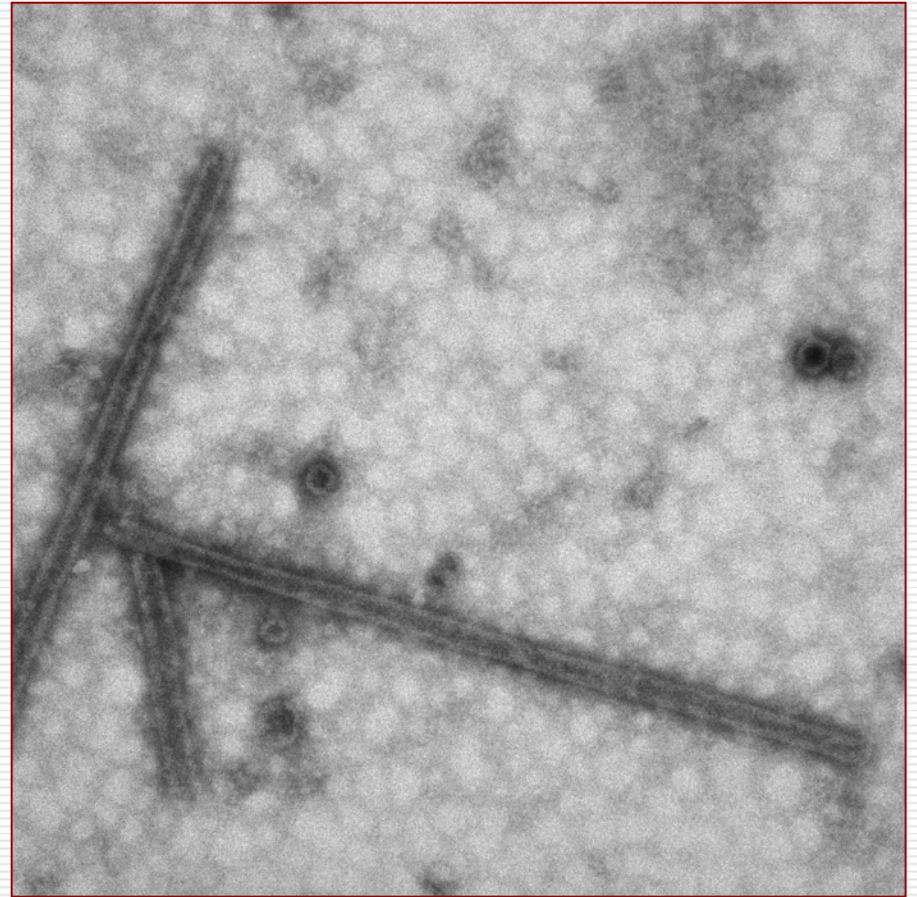
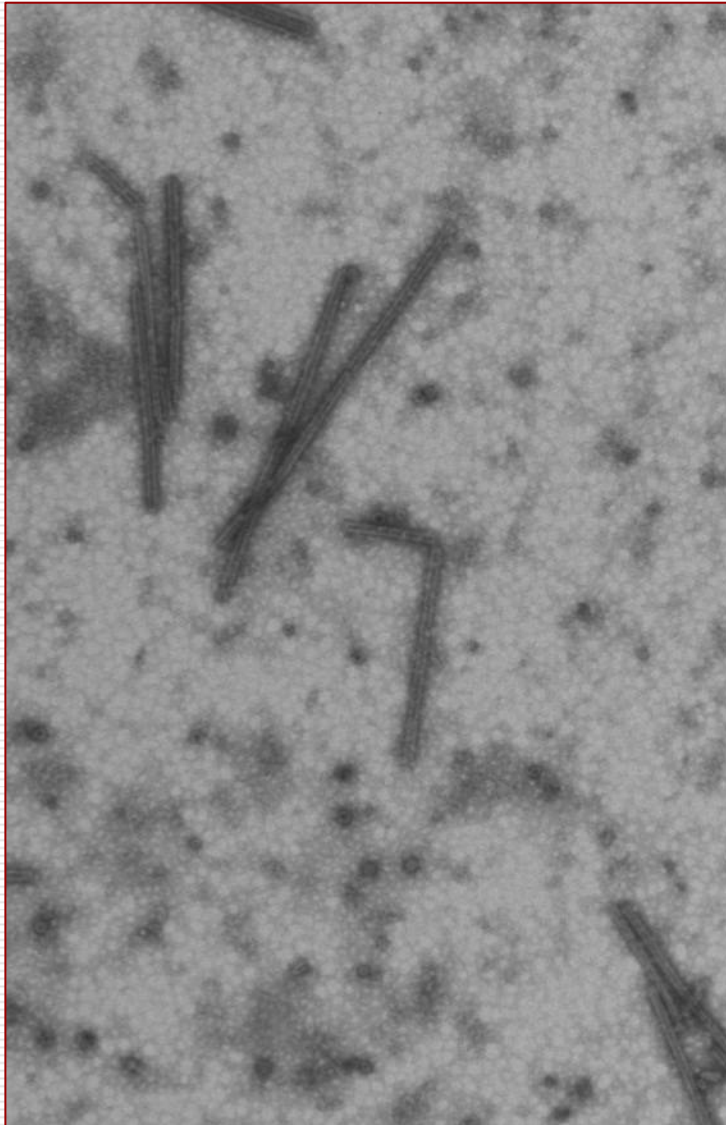


BT - BlueTongue virus (Orbivirus - Reoviridae)



From cell culture,
semipurified viral fraction

EHDV - Epizootic Haemorrhagic Disease Virus (Orbivirus - Reoviridae)



From cell culture, purified viral fraction

CHIKV - Chikungunya virus (Alphavirus - Togaviridae)

EMERGING
INFECTIOUS DISEASES®

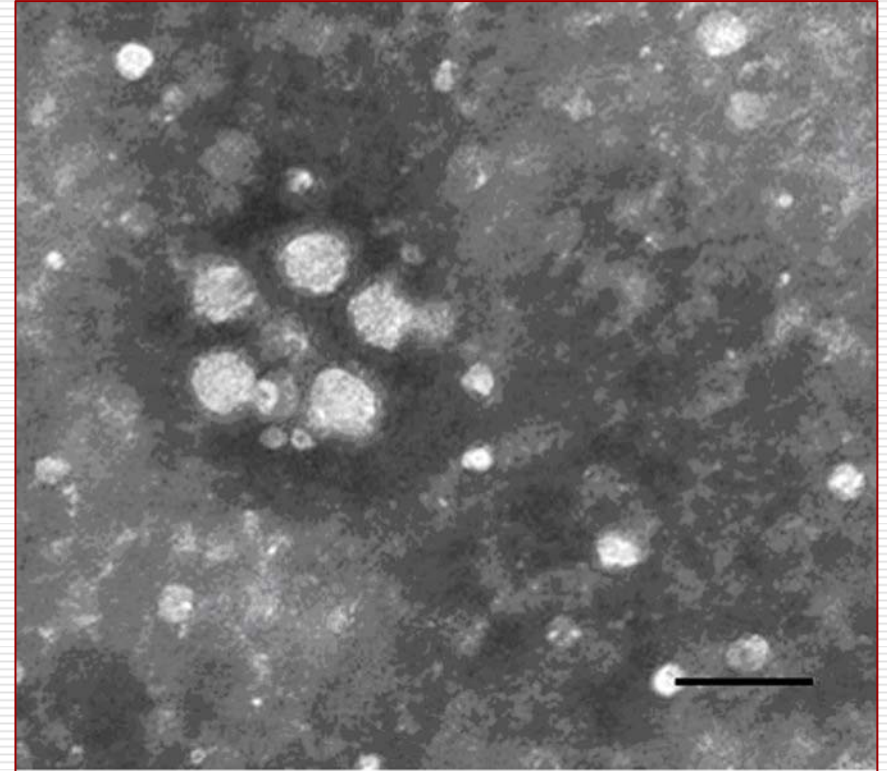
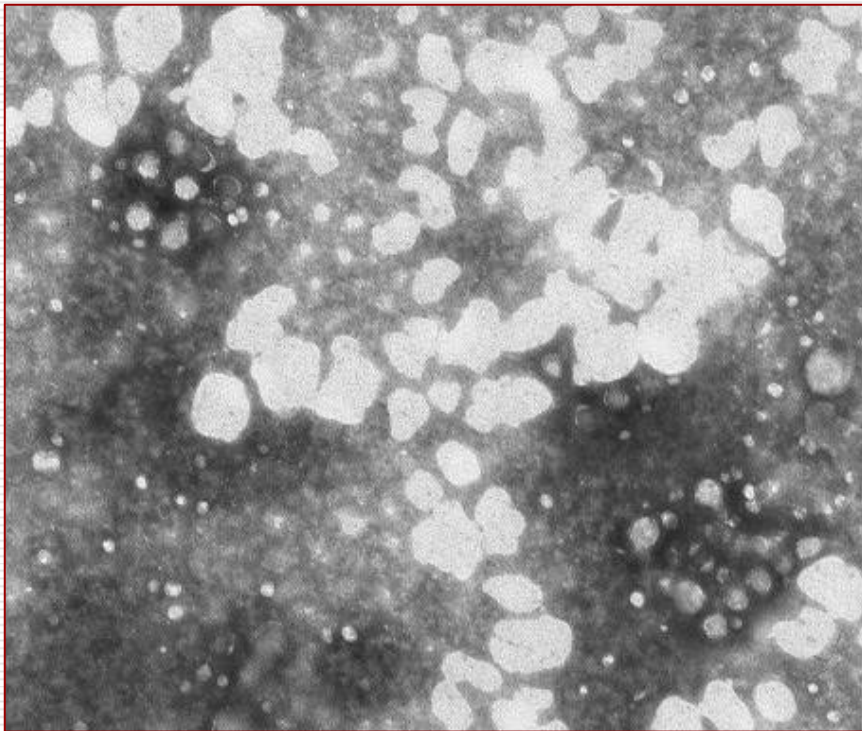


Emerg Infect Dis. 2008 May; 14(5): 852–854.
doi: [10.3201/eid1405.071144](https://doi.org/10.3201/eid1405.071144)

PMCID: PMC2600251

Chikungunya Virus in *Aedes albopictus*, Italy

Paolo Bonilauri,[✉] Romeo Bellini,[†] Mattia Calzolari,^{*} Raffaella Angelini,[‡] Luciano Venturi,[‡] Francesca Fallacara,^{*} Paolo Cordioli,^{*} Paola Angelini,[§] Claudio Venturelli,[¶] Giuseppe Meriadi,^{*} and Michele Dottori^{*}



From cell culture (VERO) with CPE

WNV - West Nile Virus (Flavivirus)

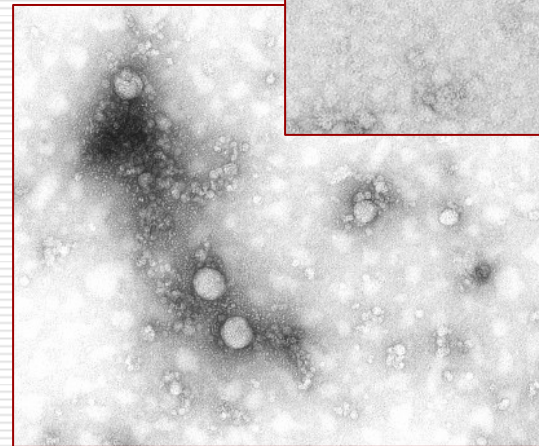
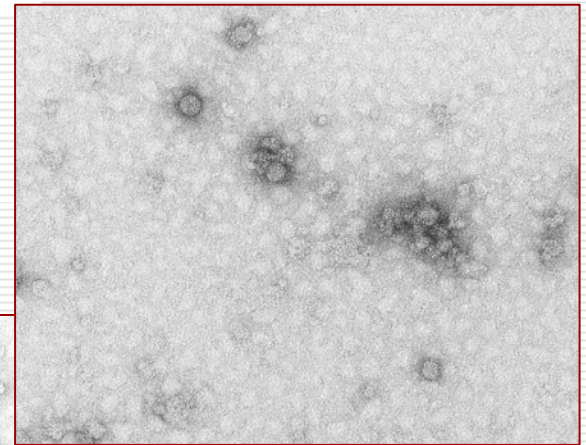
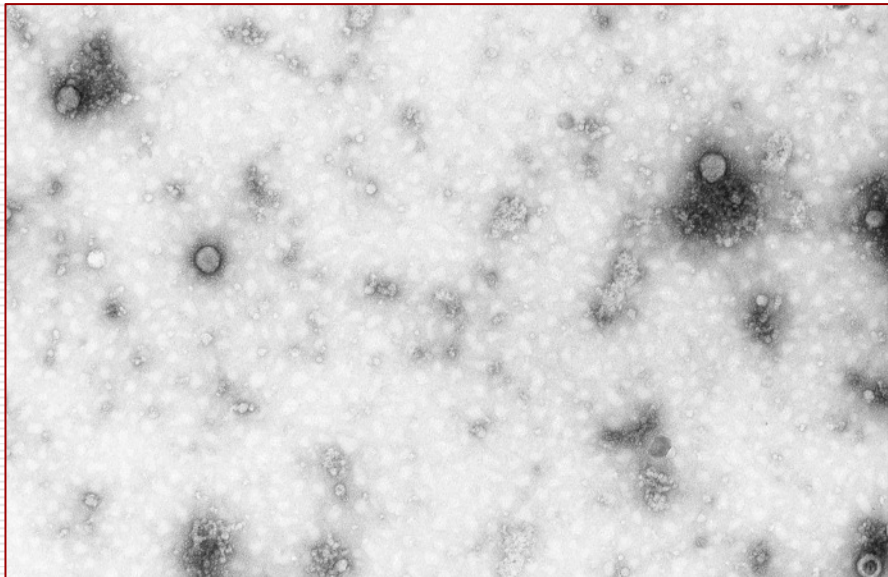
OPEN ACCESS Freely available online



Evidence of Simultaneous Circulation of West Nile and Usutu Viruses in Mosquitoes Sampled in Emilia-Romagna Region (Italy) in 2009

Mattia Calzolari^{1*}, Paolo Bonilauri¹, Romeo Bellini², Alessandro Albieri², Francesco Defilippo¹, Giulia Maioli¹, Giorgio Galletti¹, Antoni Gelati³, Ilaria Barbieri¹, Marco Tamba¹, Davide Lelli¹, Elena Carra¹, Paolo Cordioli¹, Paola Angelini⁴, Michele Dottori¹

¹ Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "B. Ubertini" (IZSLER), Brescia, Italy, ² Centro Agricoltura Ambiente "G. Nicoli" (CAA), Crevalcore, Italy, ³ Azienda USL Modena, Mirandola, Italy, ⁴ Regione Emilia-Romagna, DG Sanità e Politiche Sociali, Bologna, Italy



Mosquito viruses/1

VECTOR-BORNE AND ZOO NOTIC DISEASES
Volume 00, Number 00, 2010
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DOI: 10.1089/vbz.2009.0176

Arboviral Survey of Mosquitoes in Two Northern Italian Regions in 2007 and 2008

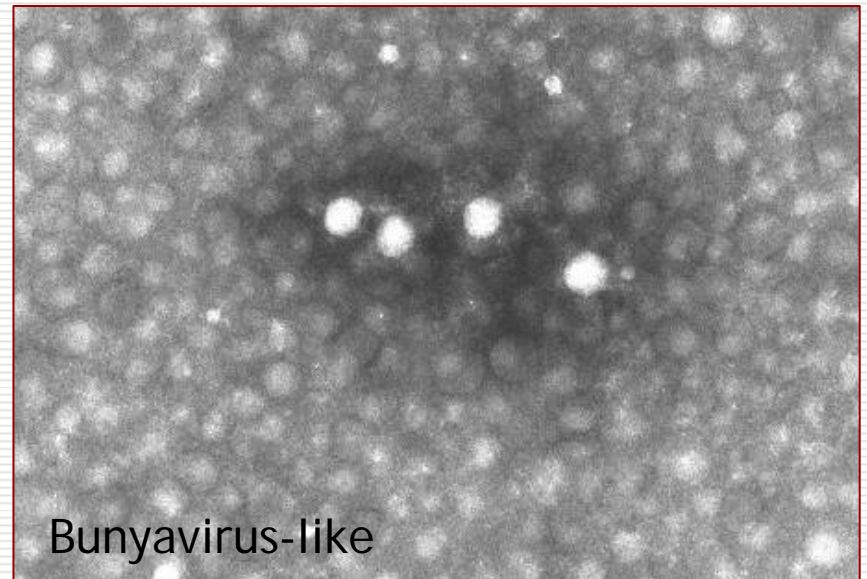
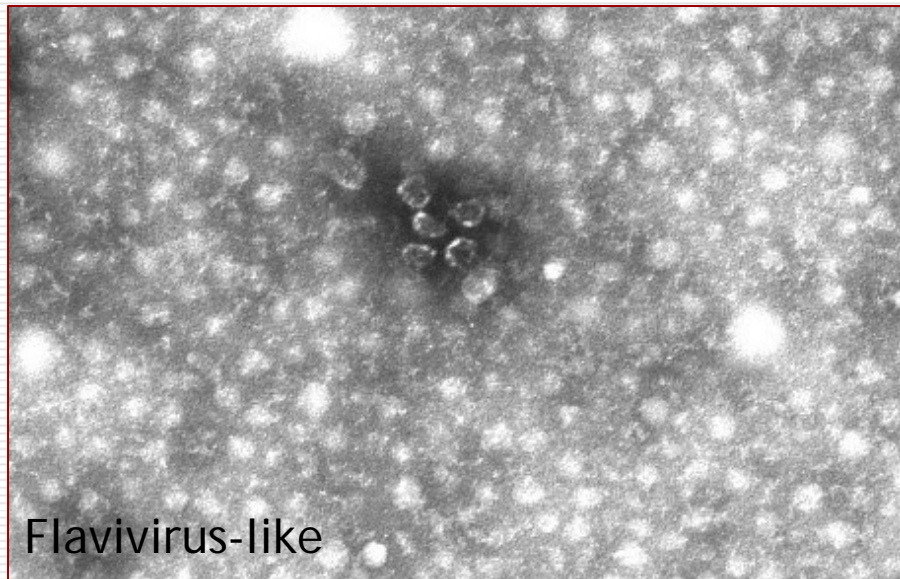
Mattia Calzolari¹, Paolo Bonilauri¹, Romeo Bellini², Marco Caimi³, Francesco Defilippo¹, Giulia Maioli¹,
Alessandro Albieri², Anna Medici², Rodolfo Veronesi², Roberto Pilani², Antonio Gelati⁴,
Paola Angelini⁵, Valentina Parco⁶, Massimo Fabbi¹, Ilaria Barbieri¹, Davide Lelli¹,
Antonio Lavazza¹, Paolo Cordioli¹, and Michele Dottori¹

PCR Results:

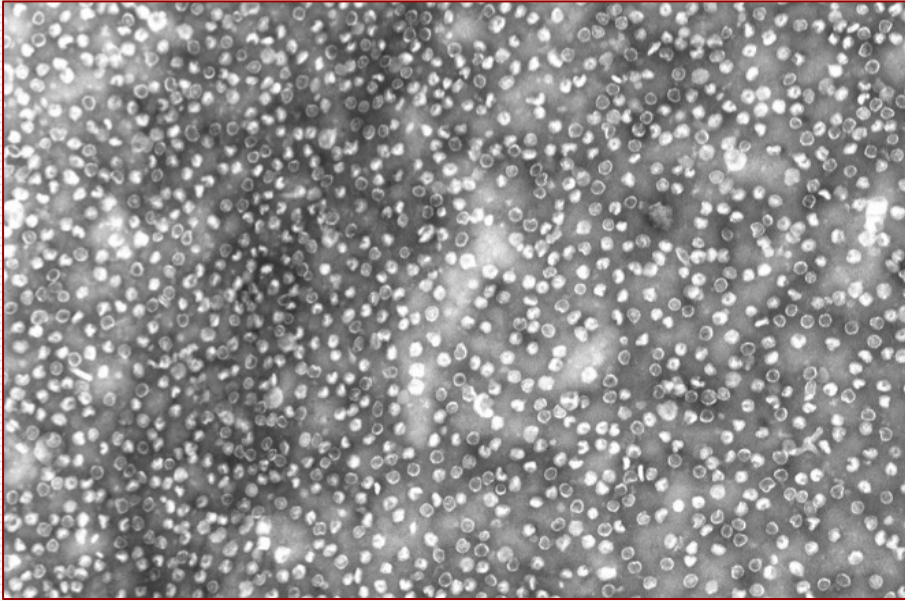
1. Detection of West Nile Virus (flavivirus)
2. Identification of RNA of Tahyna and Batai virus (bunyavirus)
3. Presence of RNA flavivirus related to mosquito flaviviruses

ME Results:

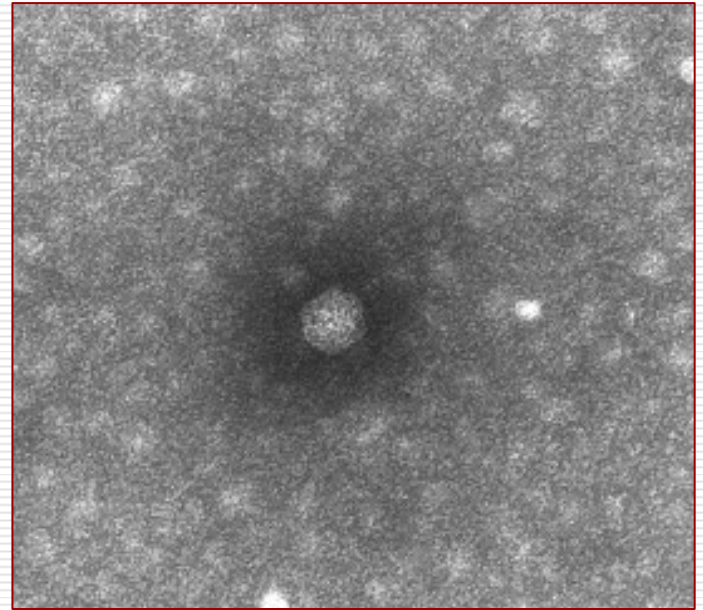
1. Reovirus/Birnalike virus
2. Flavivirus-like virus
3. Bunyavirus-like virus



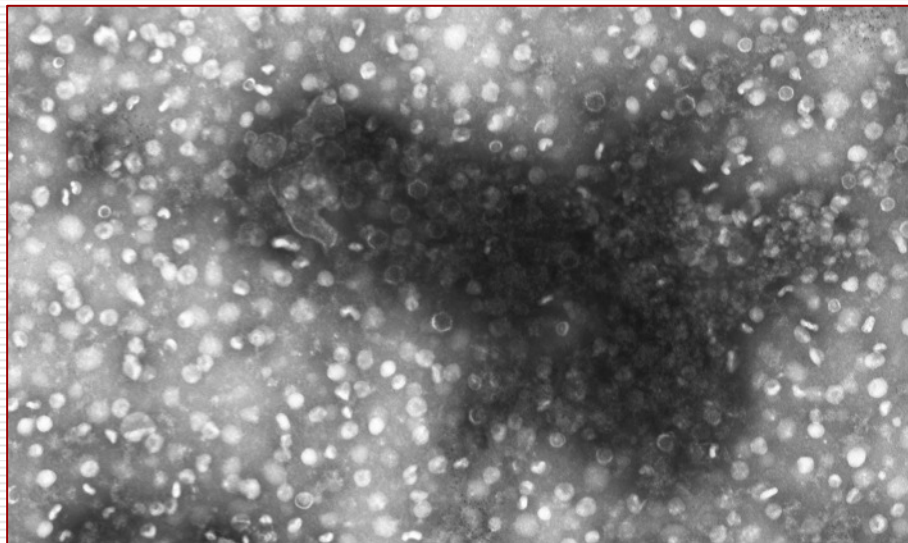
Mosquito viruses/2



Flavivirus-like

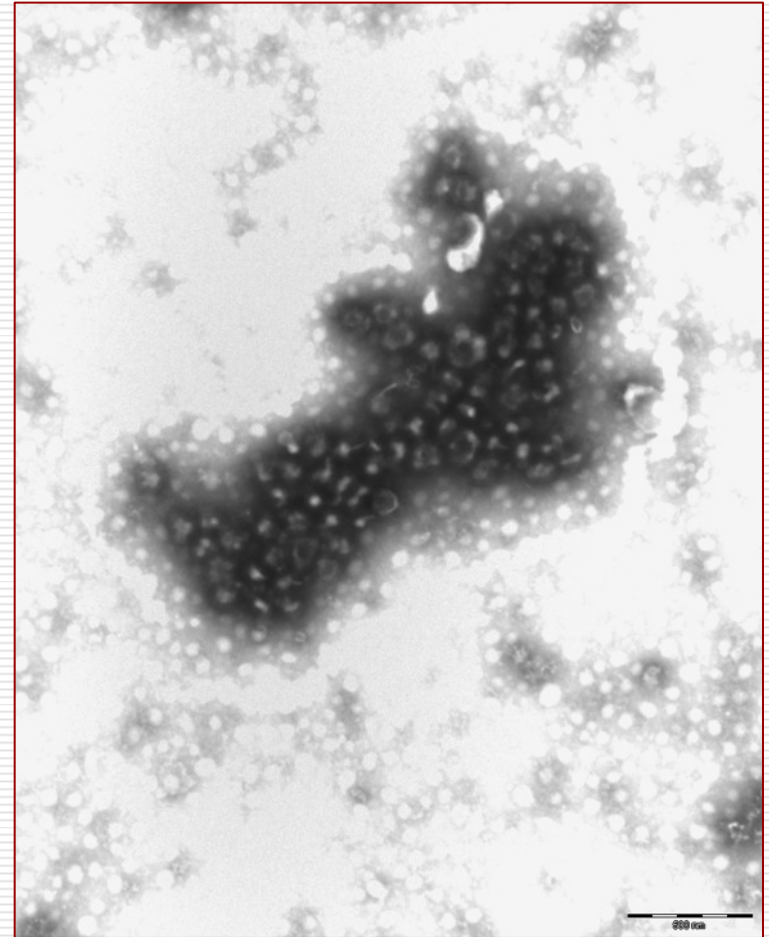
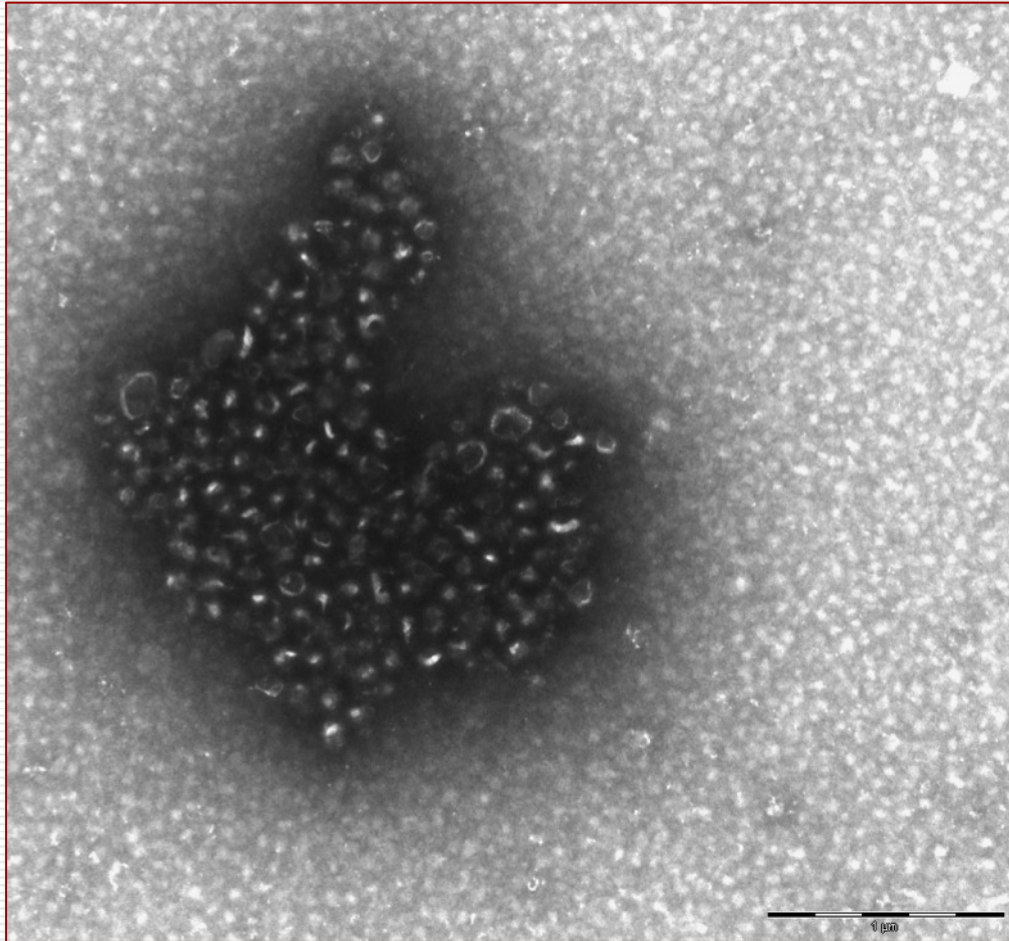


Birnavirus-like



Flavivirus-like +
Birnavirus-like

SBV- Schmallenberg virus (Orthobunyavirus)



Sucrose gradient purified virus grown on VERO cells (*viral strain was kindly furnished by Prof. T.C. Mettenleiter, FLI, Geifswald-Insel Riems, Germany*).
IEM with hyperimmune serum (given by Dr. S. Nardelli IZSve, Padova, Italy)

Viruses in minor species and wild animals

- ✓ when no other methods are available
- ✓ rare infections
- ✓ new viruses



Canary Circovirus

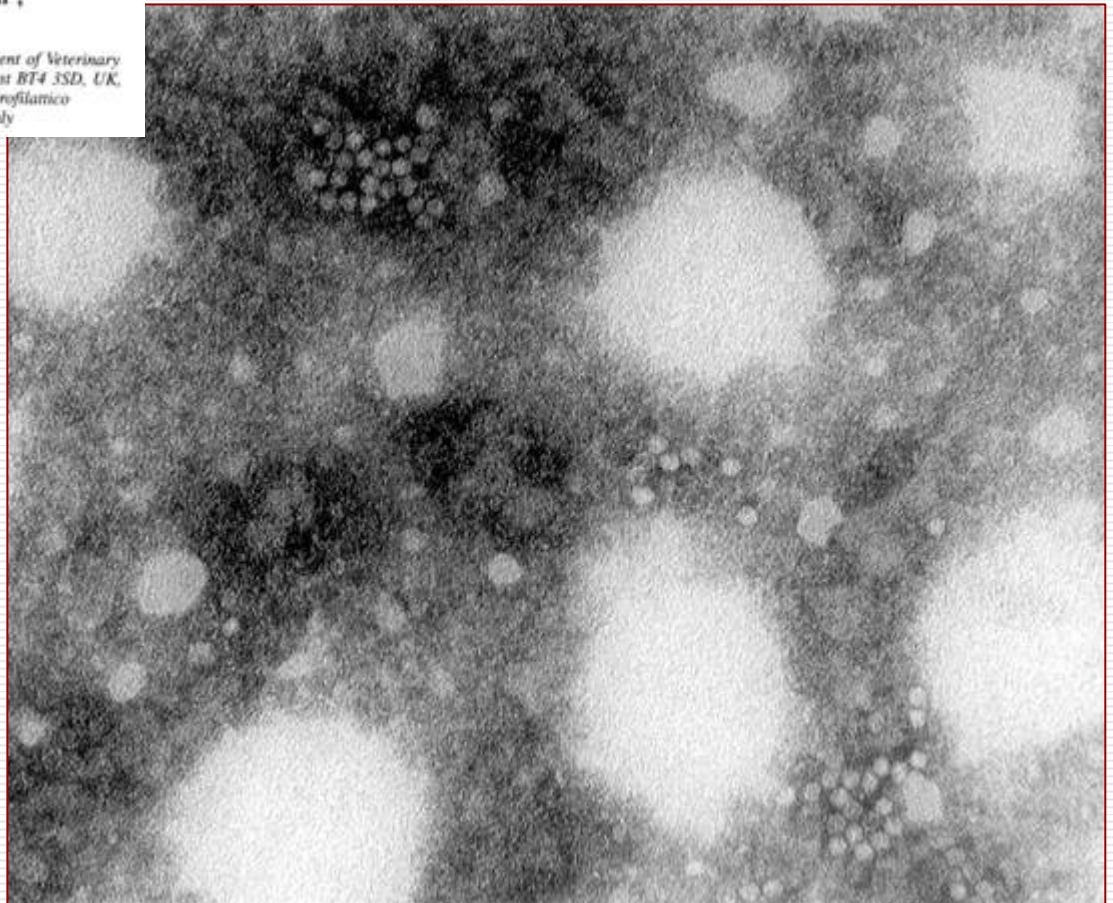
Avian Pathology (2001) **20**, 321–325



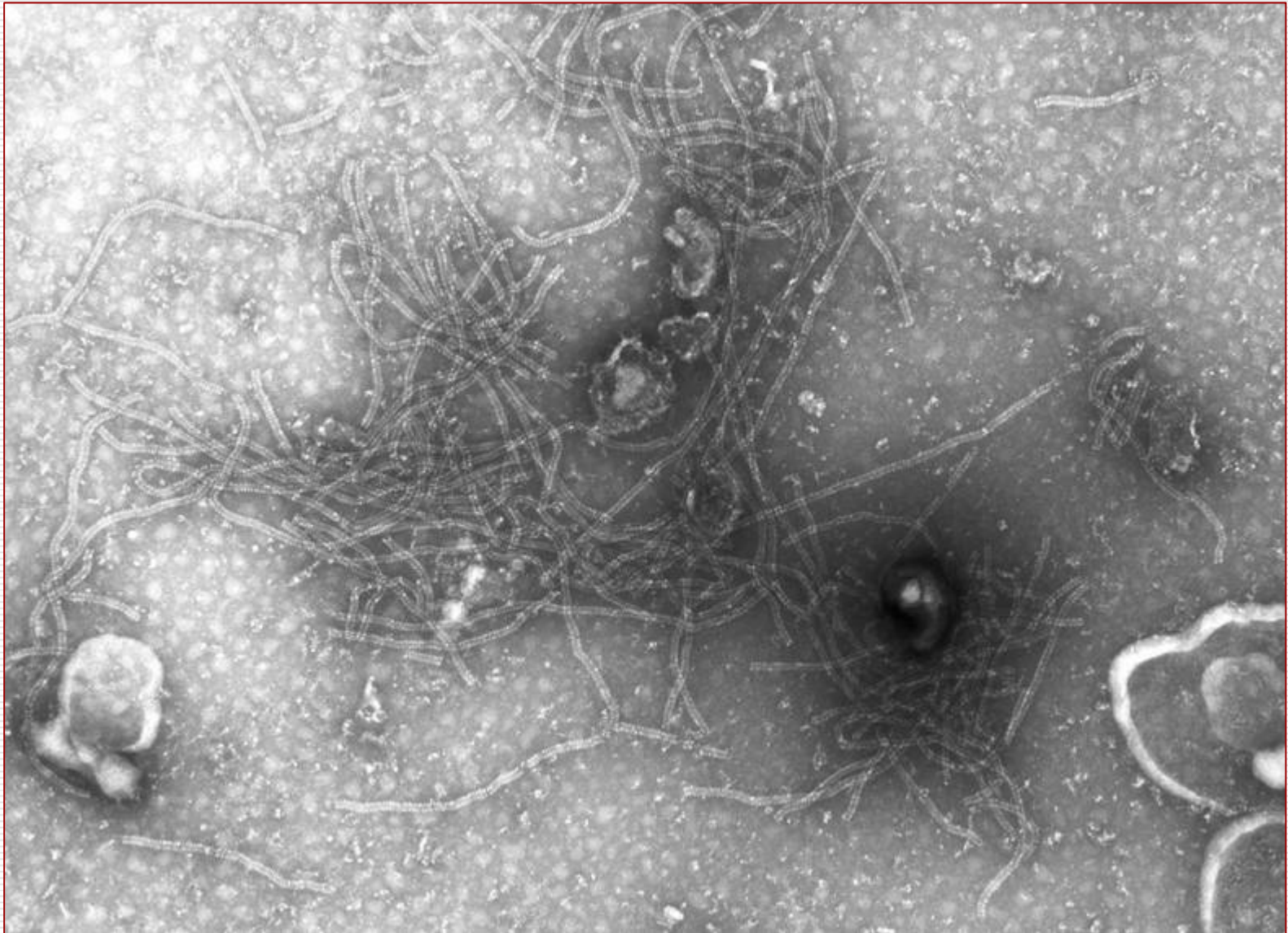
Nucleotide sequence-based identification of a novel circovirus of canaries

D. Todd¹*, J. Weston², N. W. Ball¹, B. J. Borghmans¹, J. A. Smyth¹,
L. Gelmini³ & A. Lavazza⁴

¹Department of Agriculture and Rural Development for Northern Ireland, and ²Department of Veterinary Science, the Queen's University of Belfast, Veterinary Sciences Division, Stormont, Belfast BT4 3SD, UK, and ³Diagnostic Station of Modena, and ⁴Electron Microscopy Laboratory, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Via Bianchi 9, 25124 Brescia, Italy

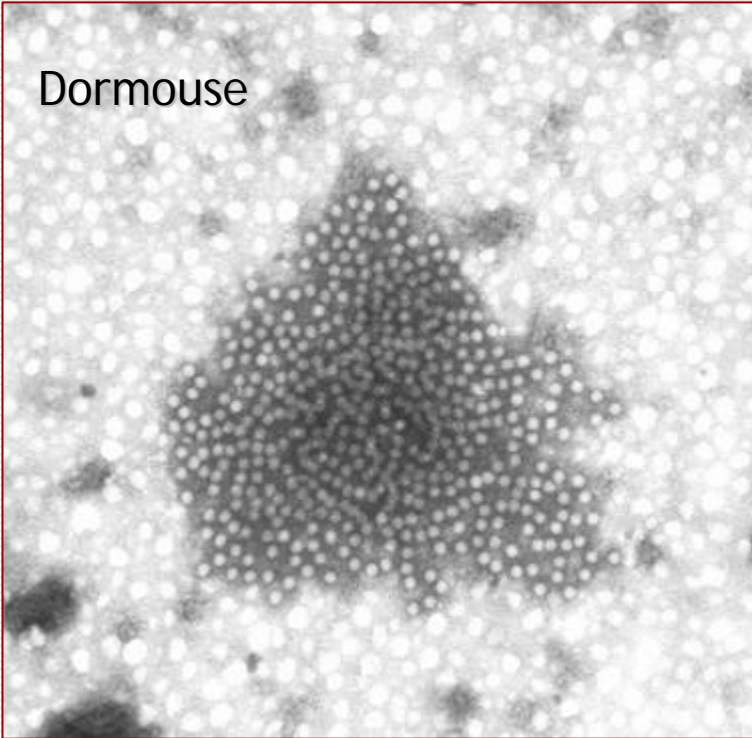


Fox Morbillivirus (Canine Distemper Virus)



Encephalomyocarditis (EMCV) virus

Dormouse



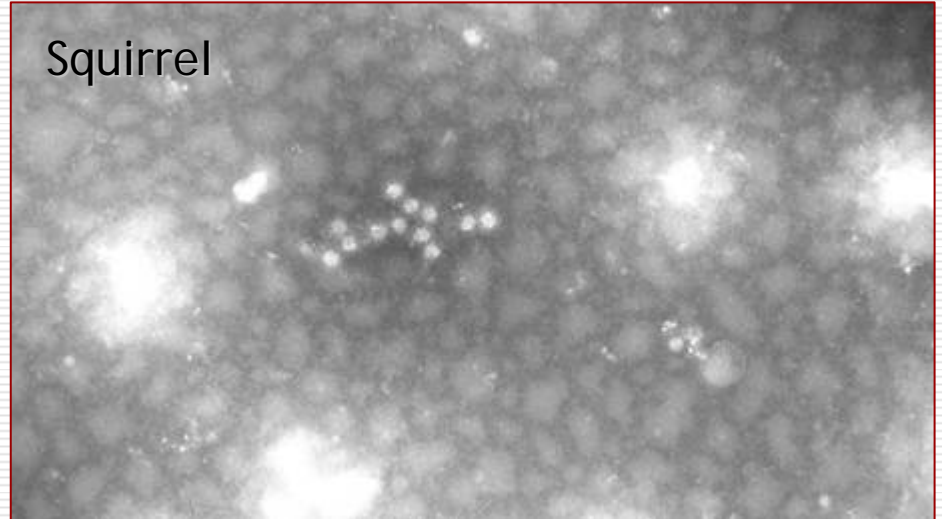
J Wildl Dis. 1995 Apr;31(2):238-42.

Isolation of encephalomyocarditis virus from dormice (*Myoxus glis*) in Italy.

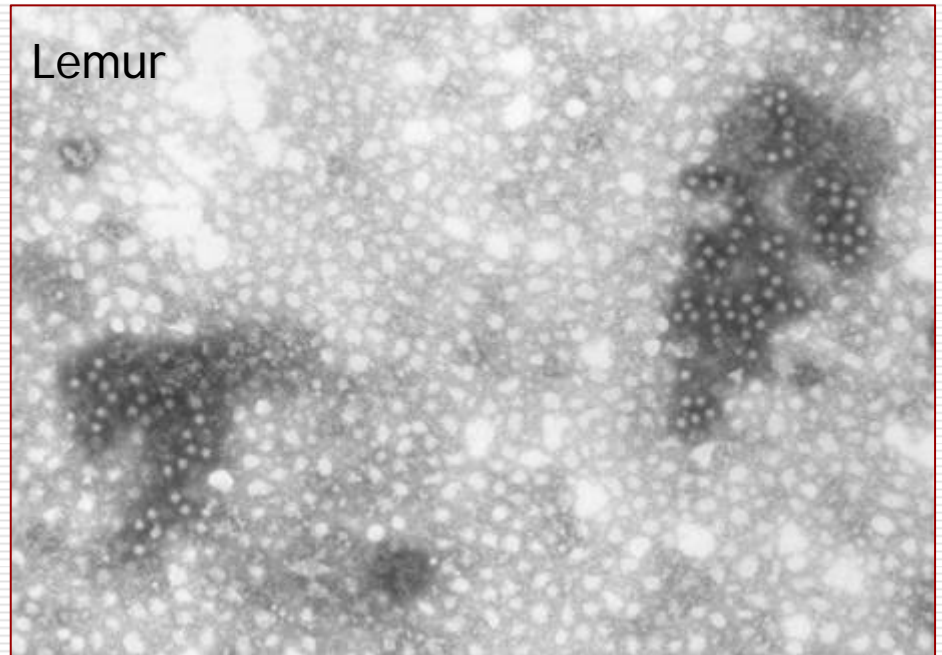
[Amaddeo D](#), [Cardeti G](#), [Autorino GL](#).

Virology Laboratory, Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Rome, Italy.

Squirrel



Lemur



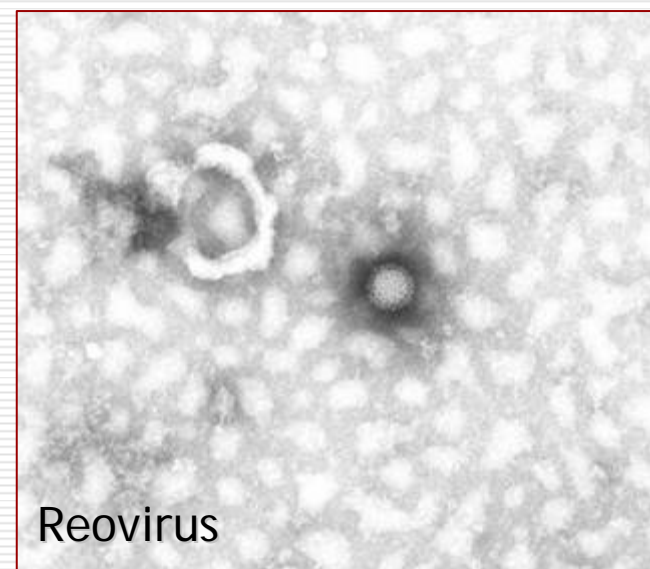
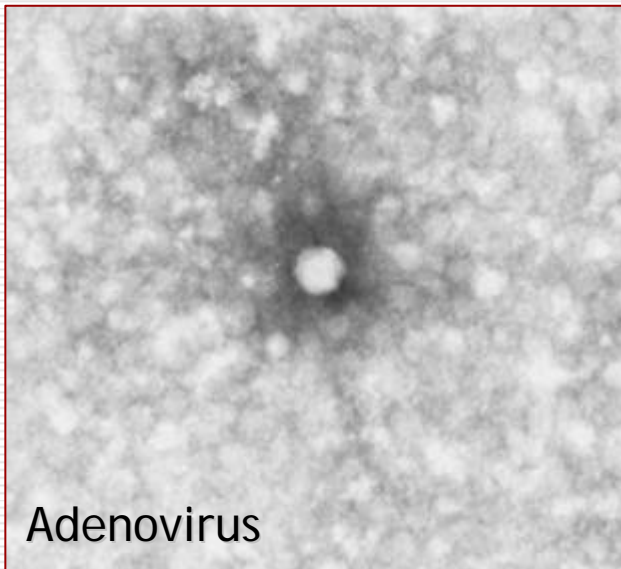
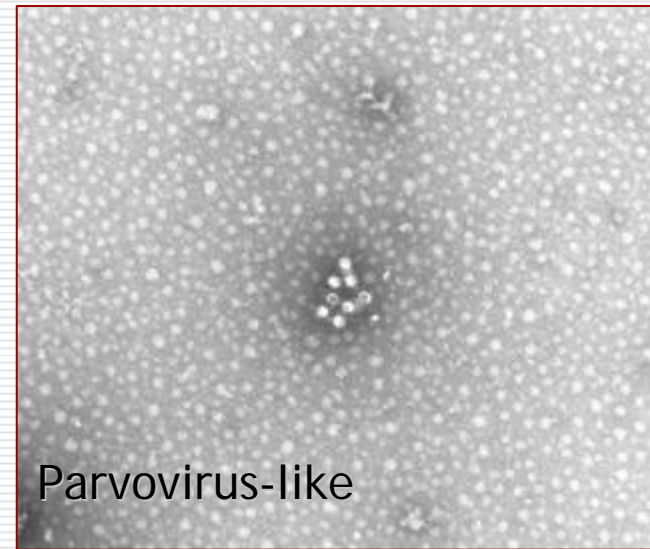
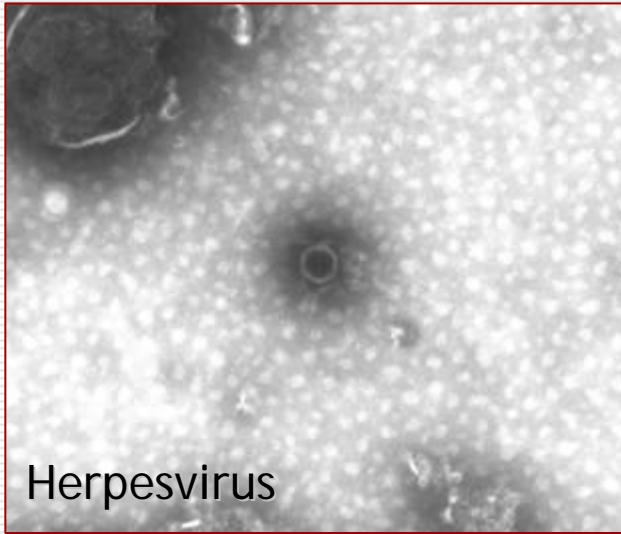
[Virology J](#). 2010 Mar 18;7:64. doi: 10.1186/1743-422X-7-64.

Encephalomyocarditis virus infection in an Italian zoo.

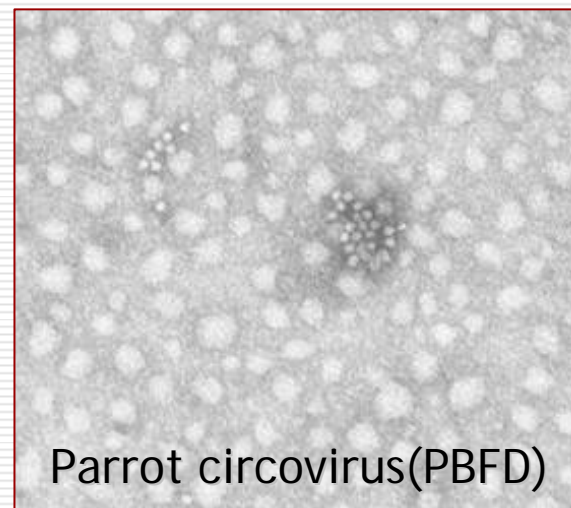
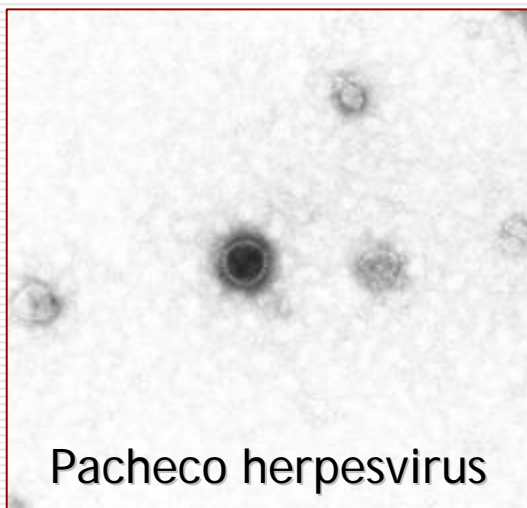
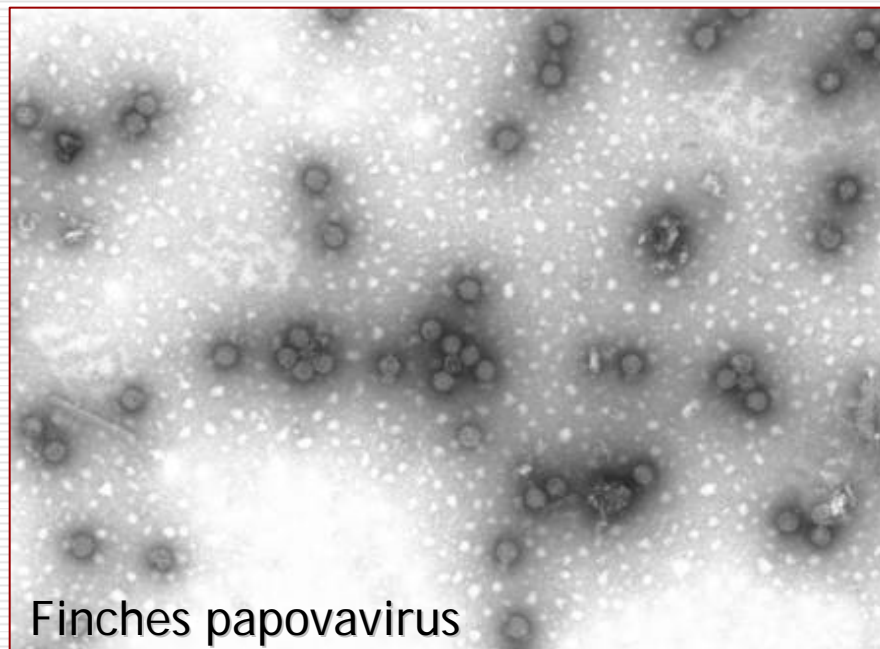
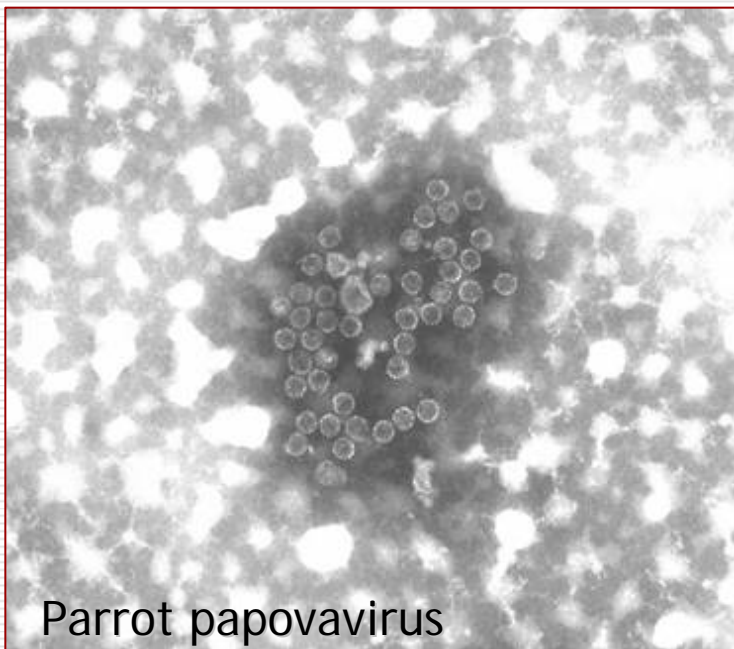
[Canelli E](#), [Luppi A](#), [Lavazza A](#), [Lelli D](#), [Sozzi E](#), [Martin AM](#), [Gelmetti D](#), [Pascotto E](#), [Sandri C](#), [Magnone V](#), [Cordioli P](#).

Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna B Ubertini (IZSLER), Via Bianchi, 7/9 - 25124 Brescia, Italy

Turtles



Parrots and Finches

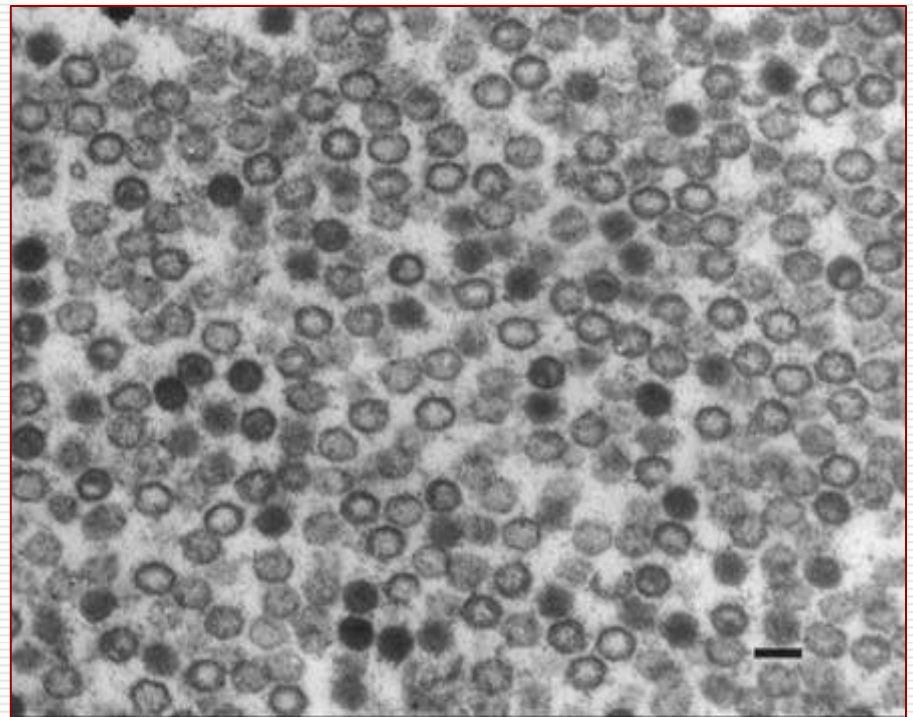
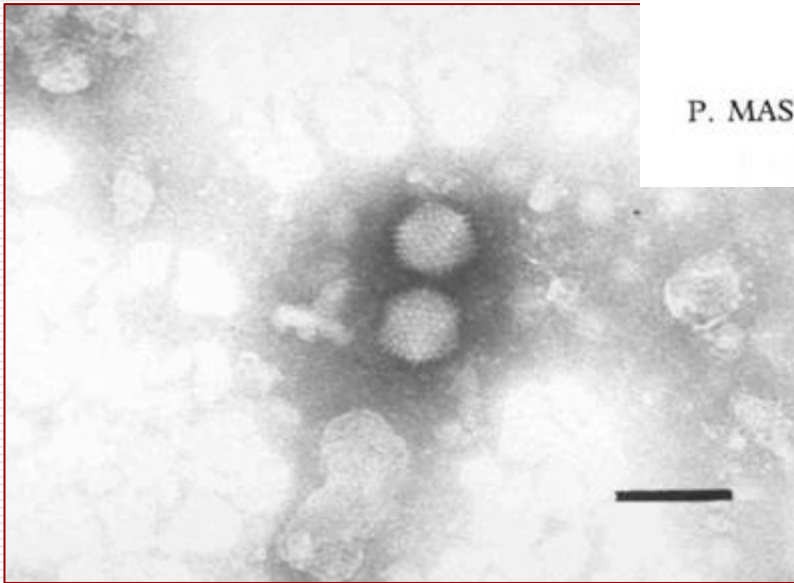


Aviadenovirus (type 2) in guinea fowl

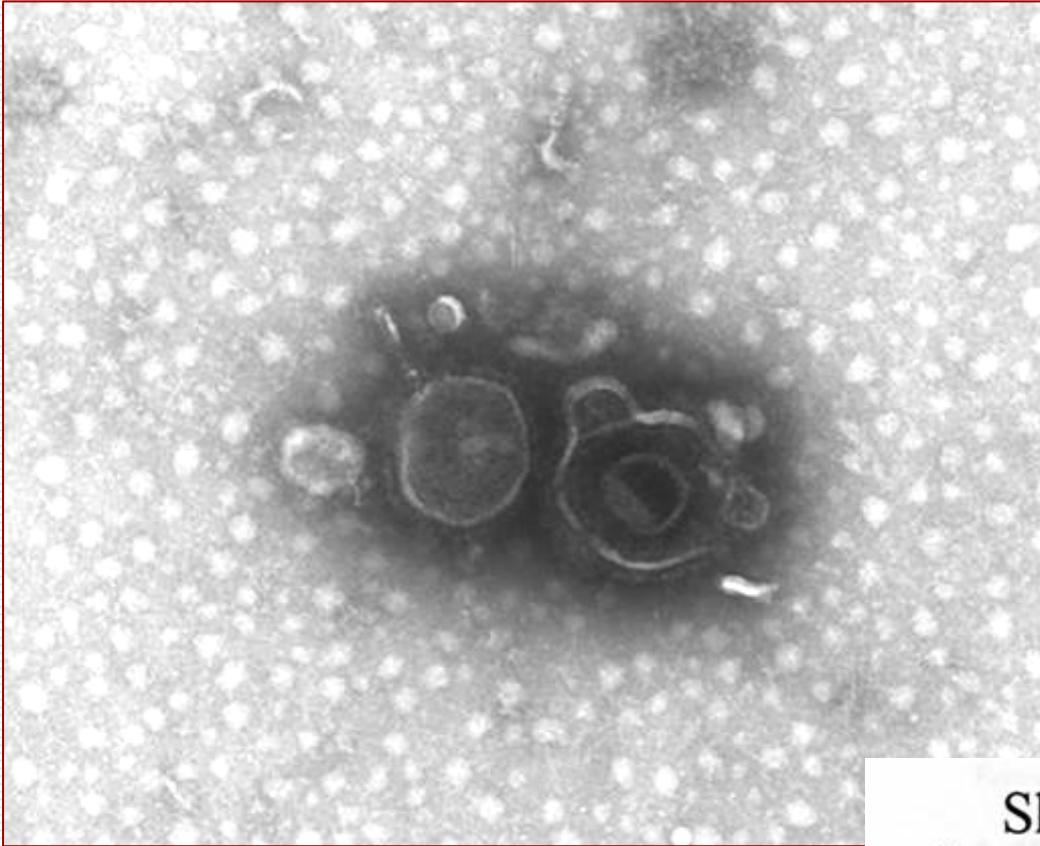
Avian Pathology (1995) 24, 227-237

Adenovirus-associated haemorrhagic disease in guinea fowl

P. MASSI¹, D. GELMETTI¹, G. SIRONI², M. DOTTORI¹, A. LAVAZZA¹
& S. PASCUCCHI¹



Frog herpesvirus



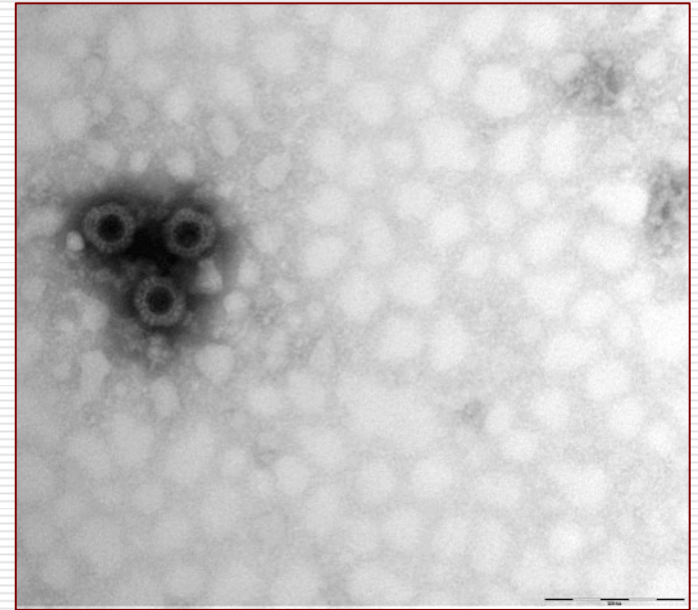
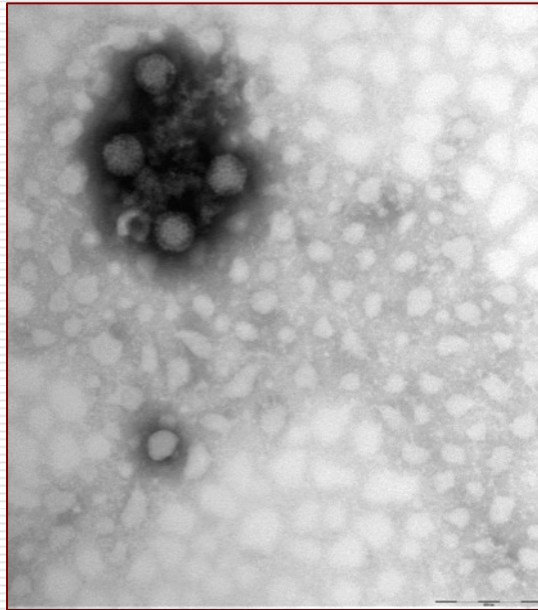
Skin lesions associated with
herpesvirus-like particles in frogs
(*Rana dalmatina*)

R. Bennati, M. Bonetti, A. Lavazza, D. Gelmetti

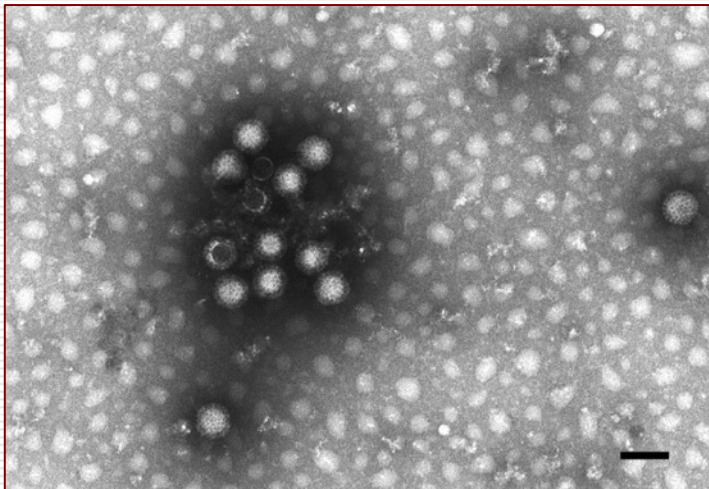
Veterinary Record (1994) 135, 625-626

Reoviridae in wild animals

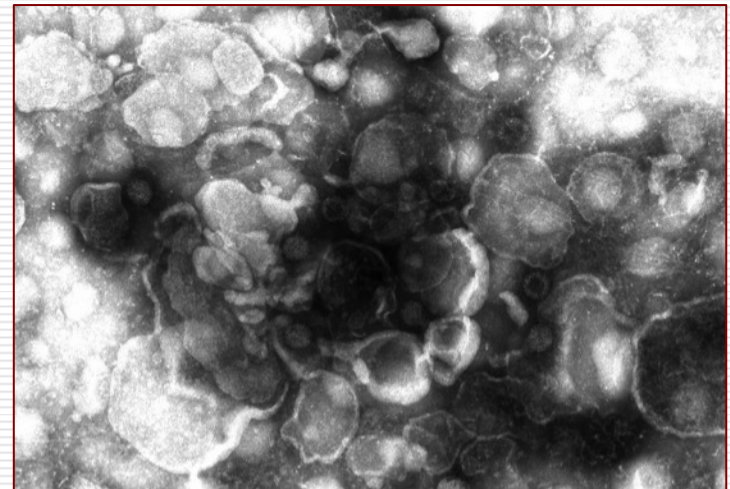
Reovirus-like
in wild boar
(after isolation
on cell culture)



Reovirus in bats



Rotavirus in a fox



Diagnosis of Fish viruses

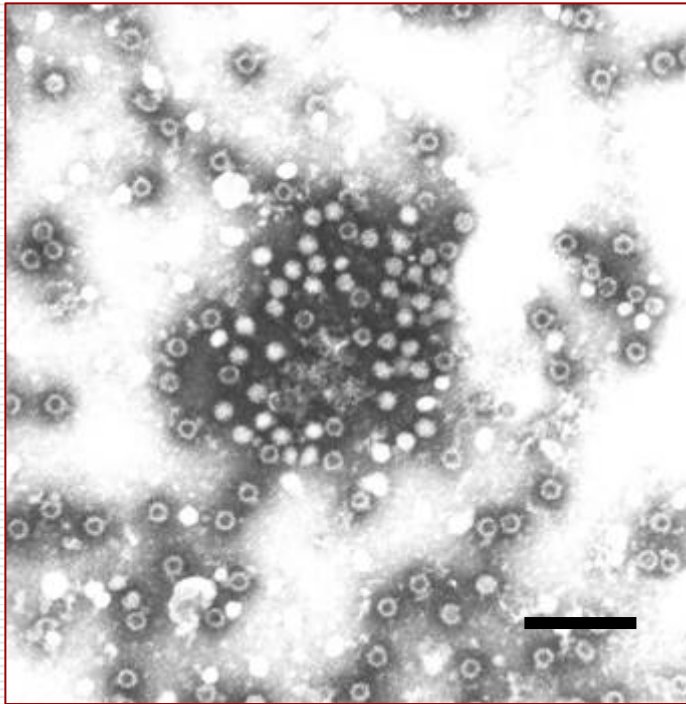
- ✕ Directly from pathological materials (gill, skin and internal organs)



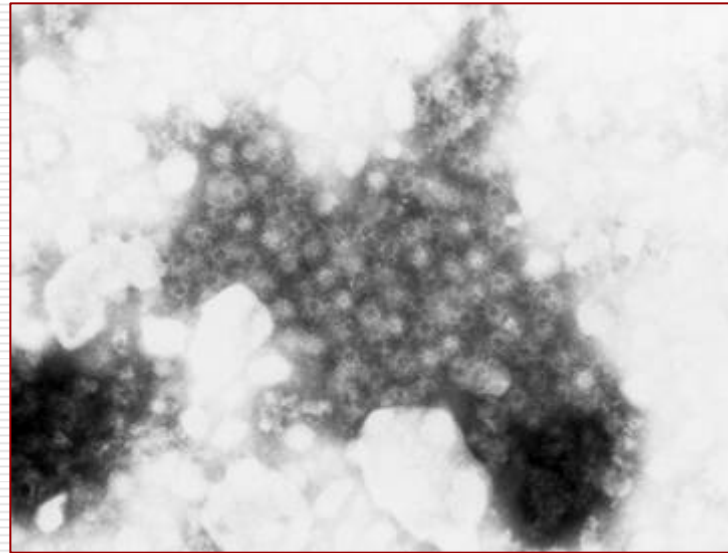
- ✕ From cell culture supernatants/lysates



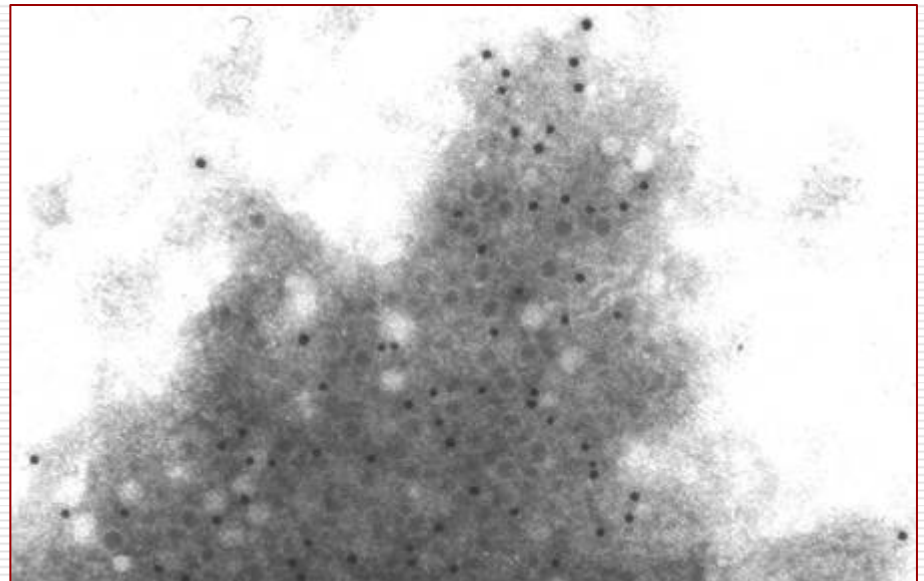
Betanodavirus



Purified



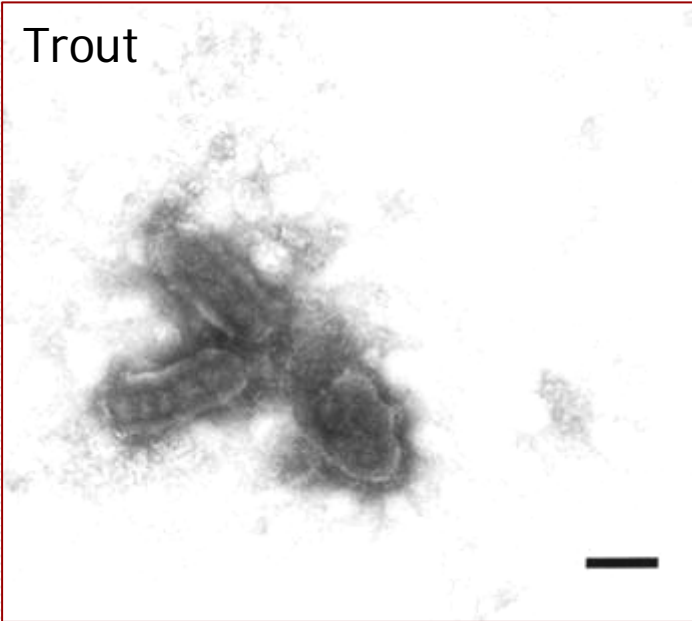
IEM



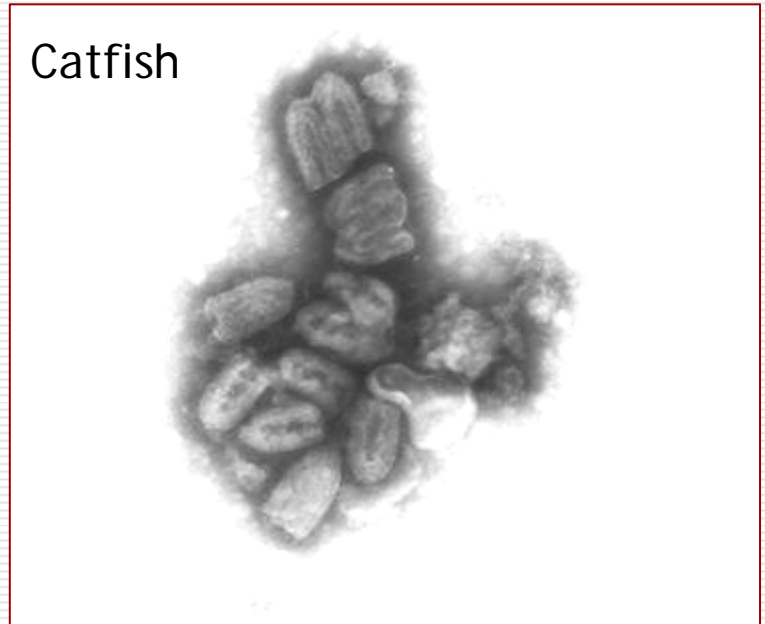
Immunogold

Rhabdovirus

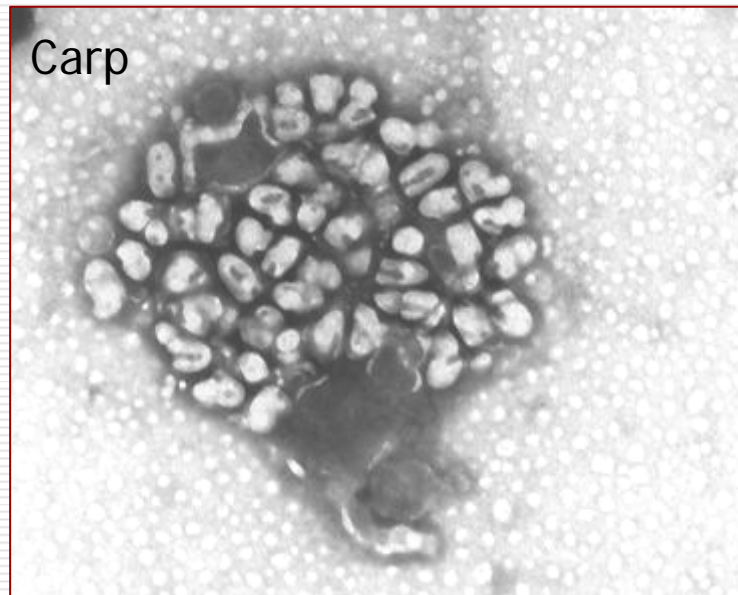
Trout



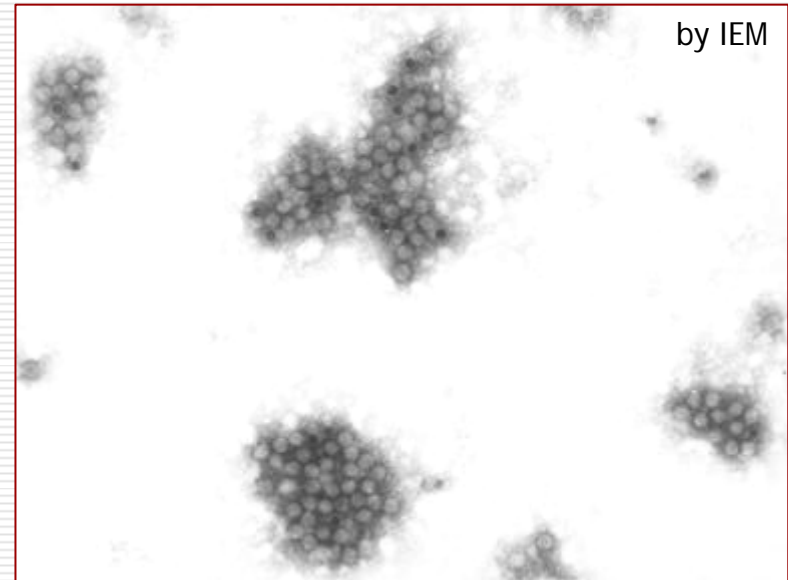
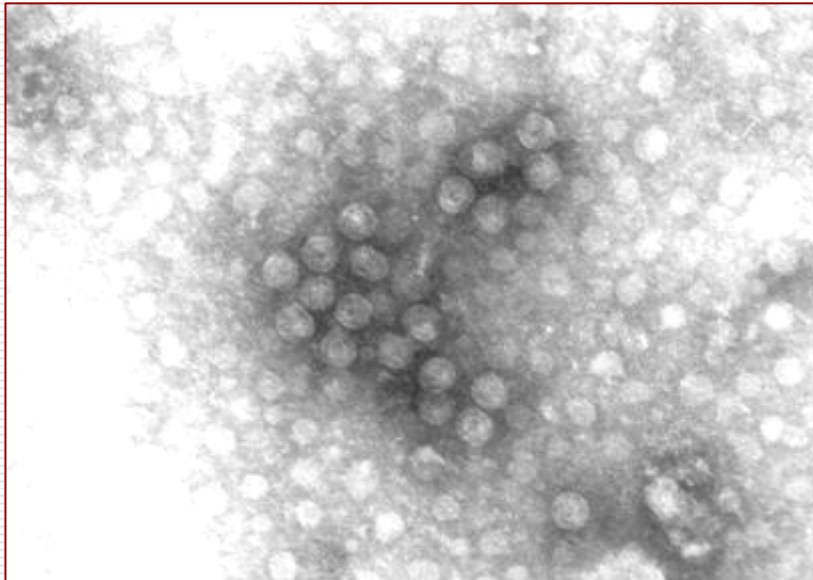
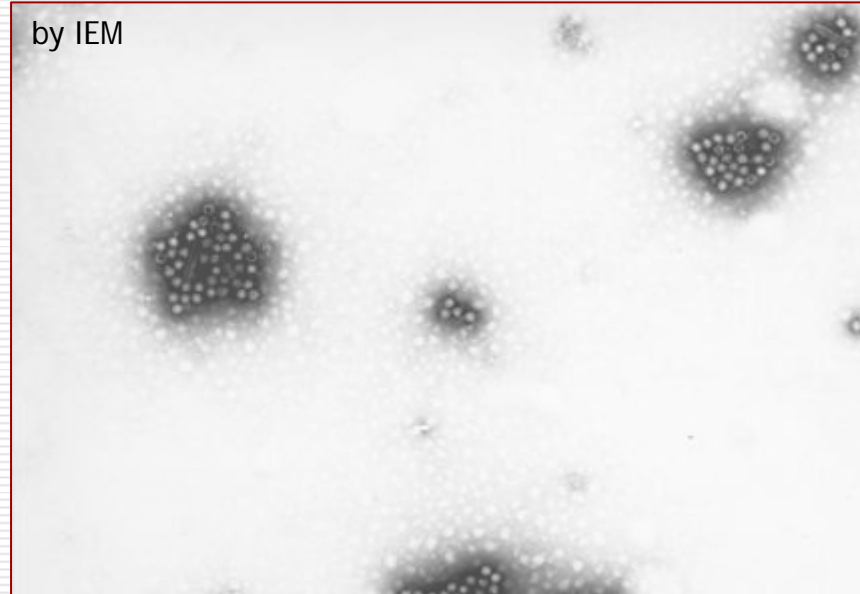
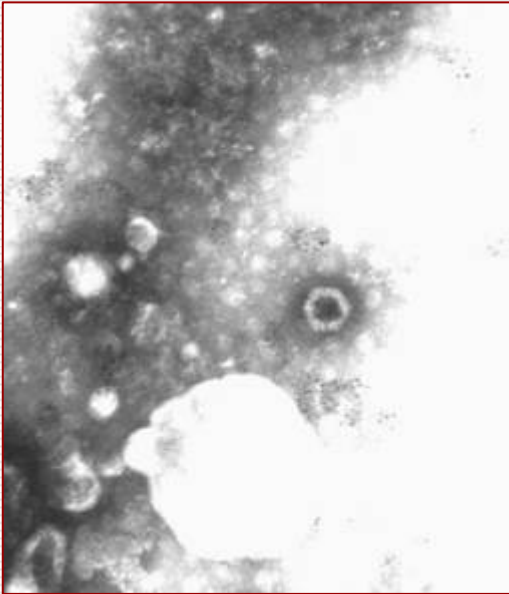
Catfish



Carp

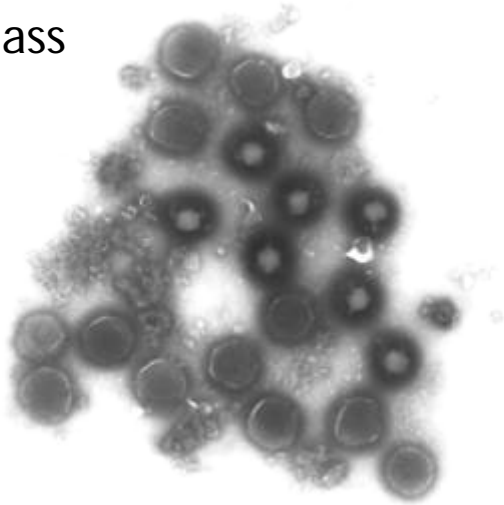


Birnavirus (IPN) in trout

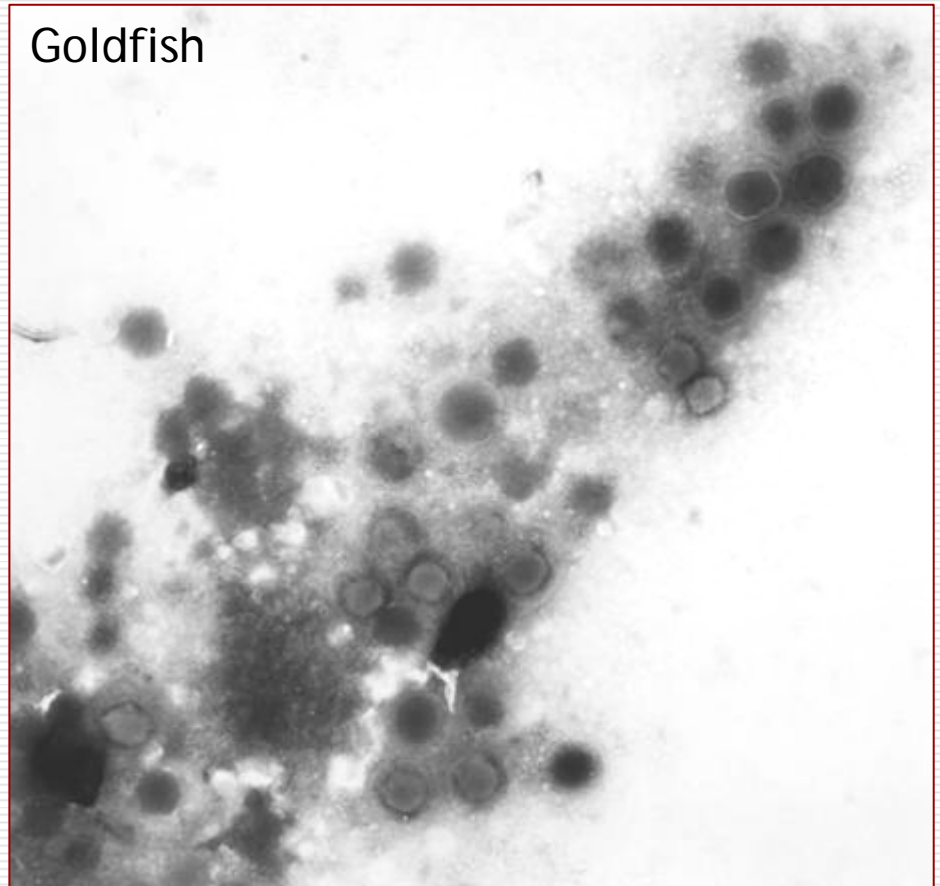


Iridovirus

Seabass

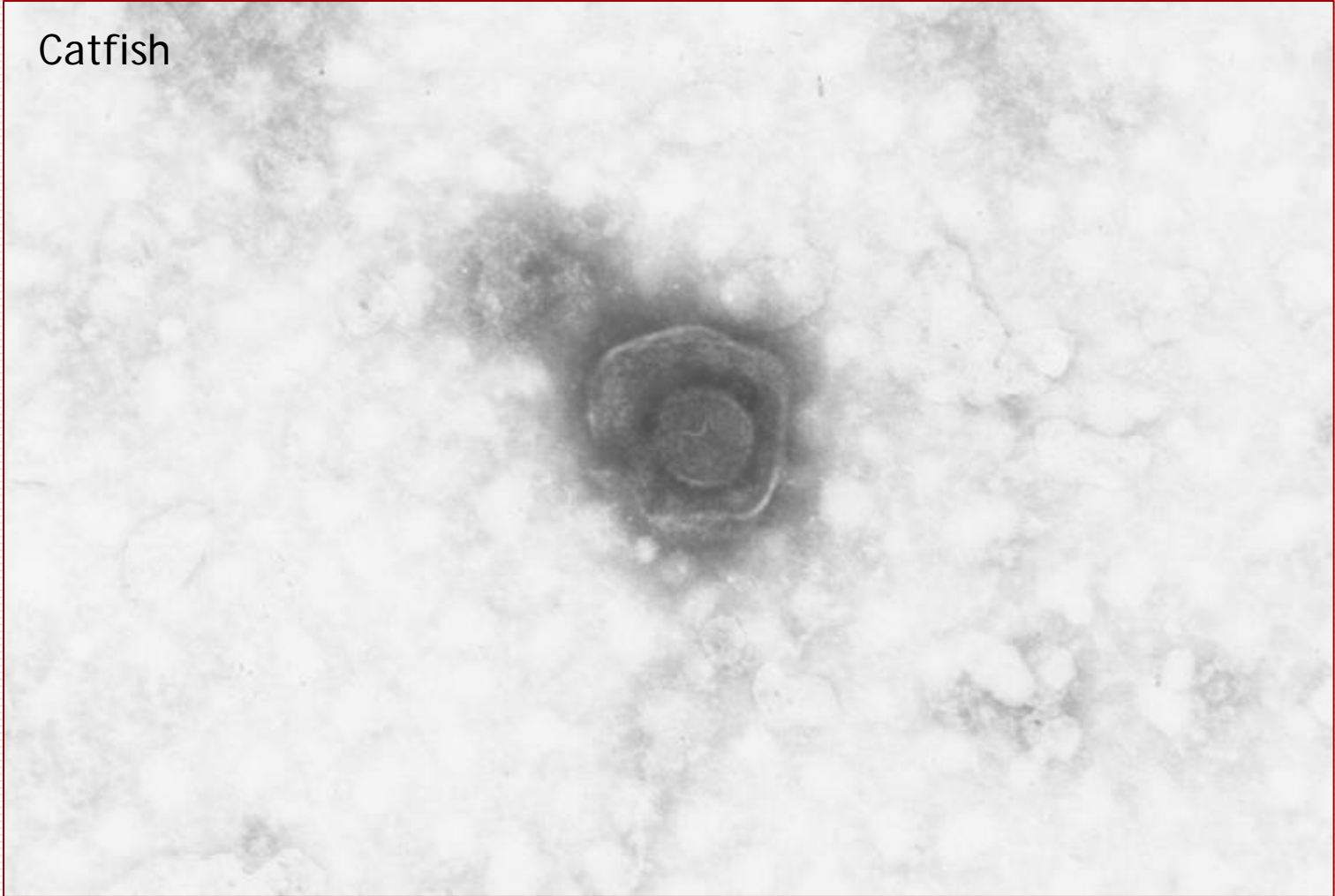


Goldfish



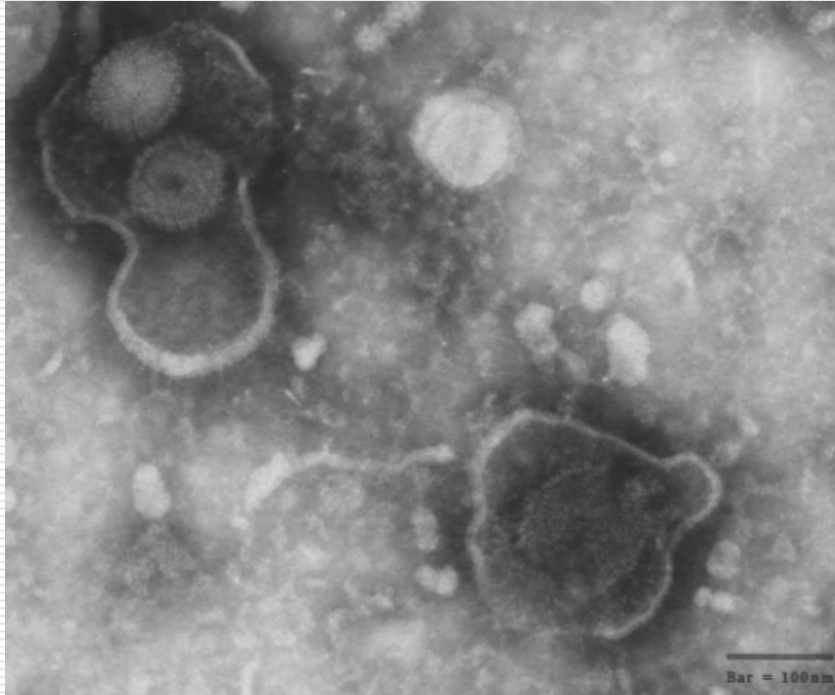
Herpesvirus

Catfish



Cyprinid Herpesvirus-2 (CyHV-2)

Carassius carassius




J Fish Dis. 2013 Oct;36(10):823-30. doi: 10.1111/jfd.12048. Epub 2013 Mar 11.

Detection of Cyprinid herpesvirus 2 in association with an *Aeromonas sobria* infection of *Carassius carassius* (L.), in Italy.

Fichi G, Cardeti G, Cocumelli C, Vendramin N, Toffan A, Eleni C, Siemoni N, Fischetti R, Susini F.

Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Rome, Italy.

Diagnosis of Honeybee viruses

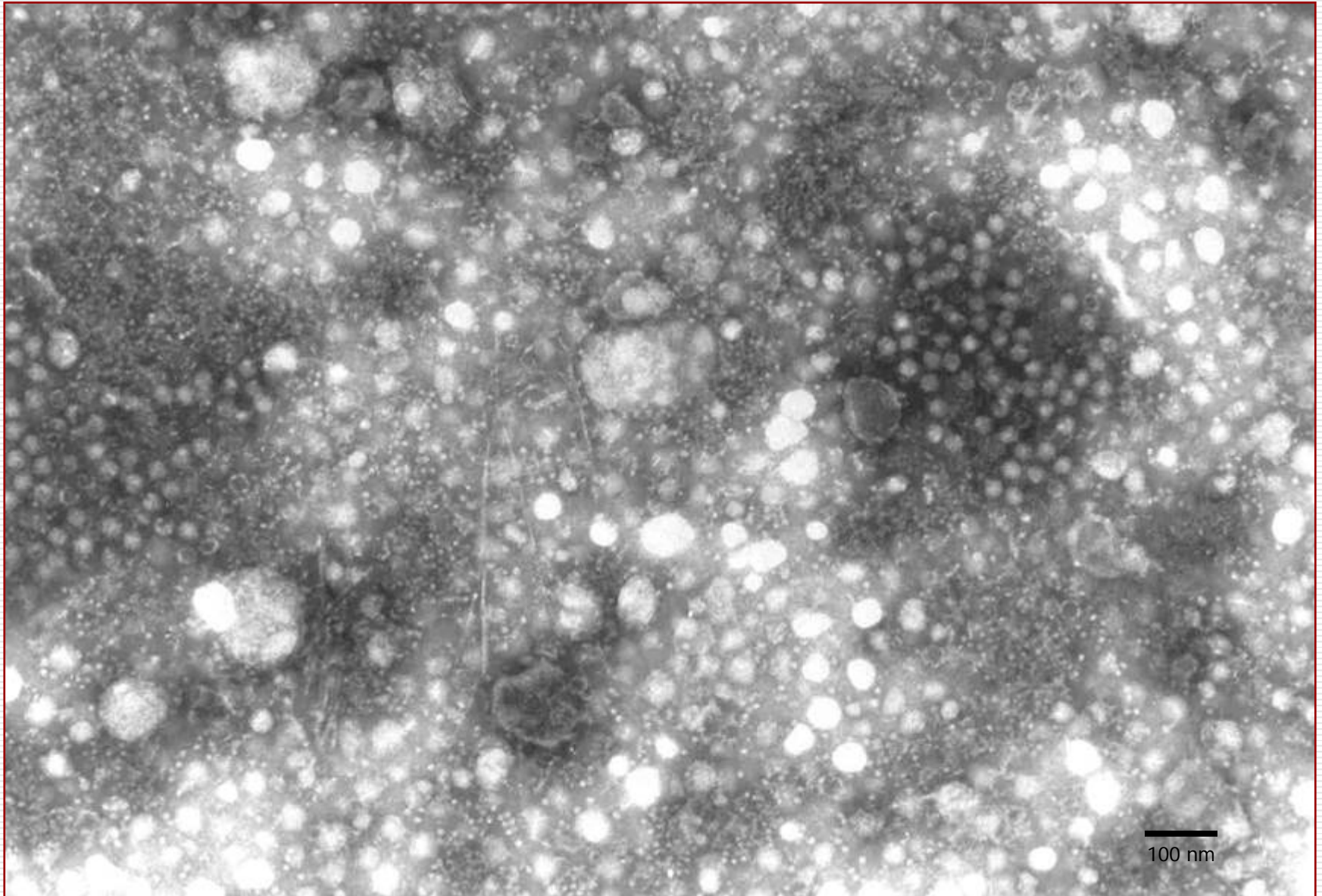
- 
- Initially only by AGID (till '90)
 - EM employed as “multitask” method when specific sera were unavailable (from early '90)
 - Wider use of molecular methods (PCR and RealTime-PCR) (starting from 2000) → *elective method*

But.....

- Often latent infections (nucleic acid vs mature particles)
- Correct interpretation of the result of very sensitive methods (latent infection vs overt disease)
- New viruses and new variants
- Virus association

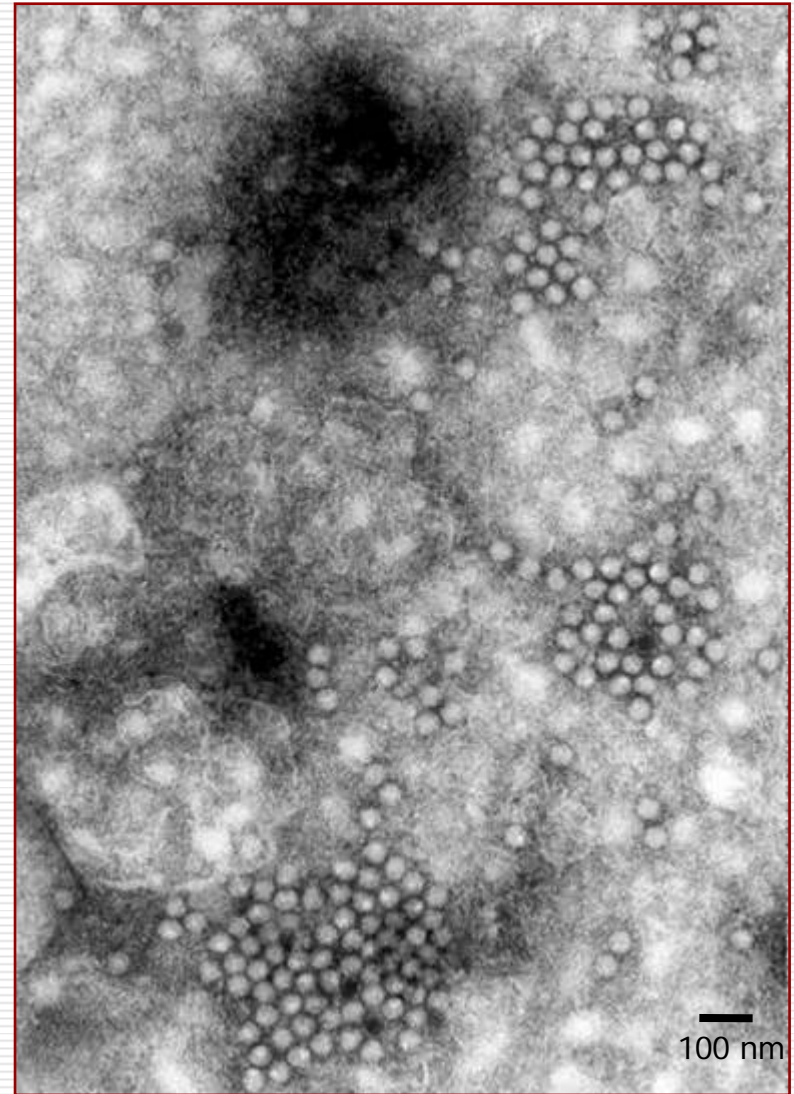
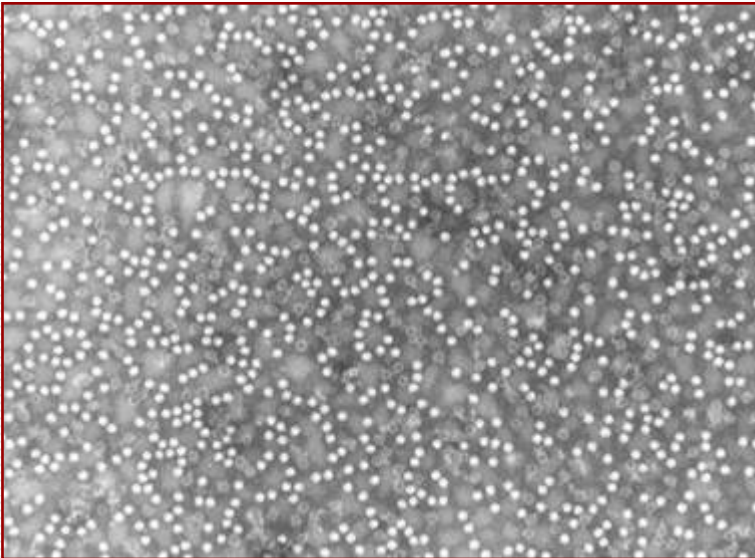


Acute Bee Paralysis Virus (ABPV) - IEM

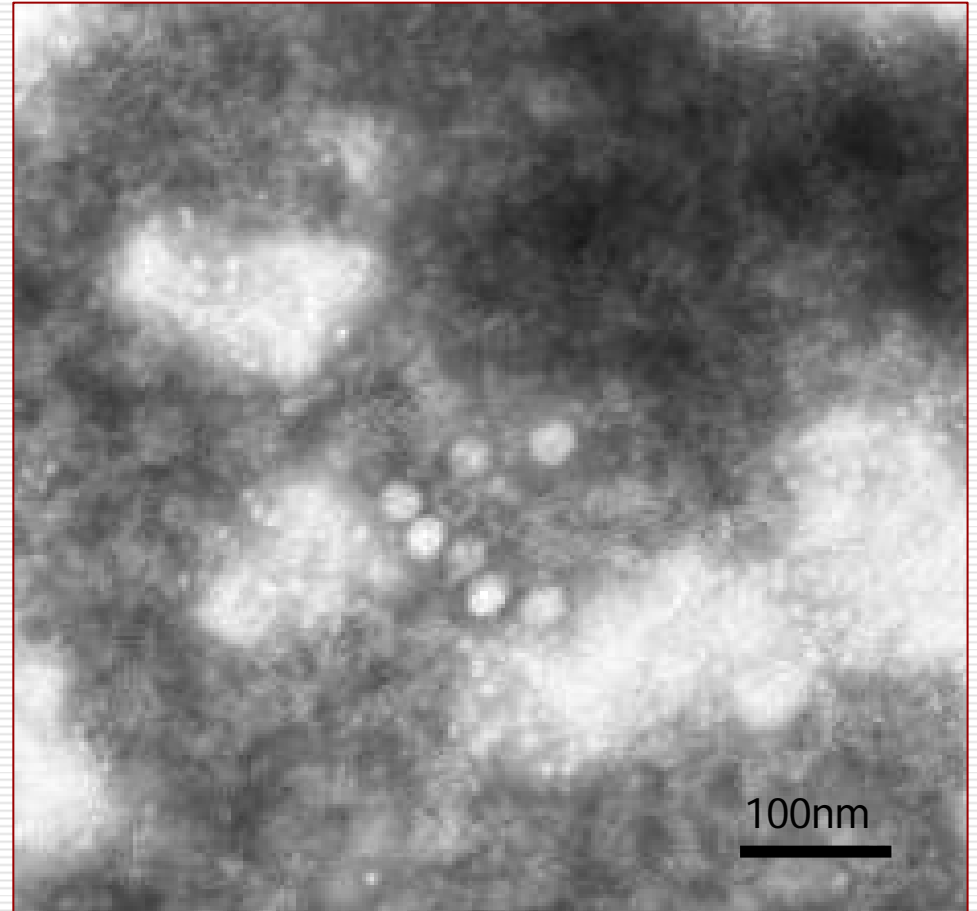


100 nm

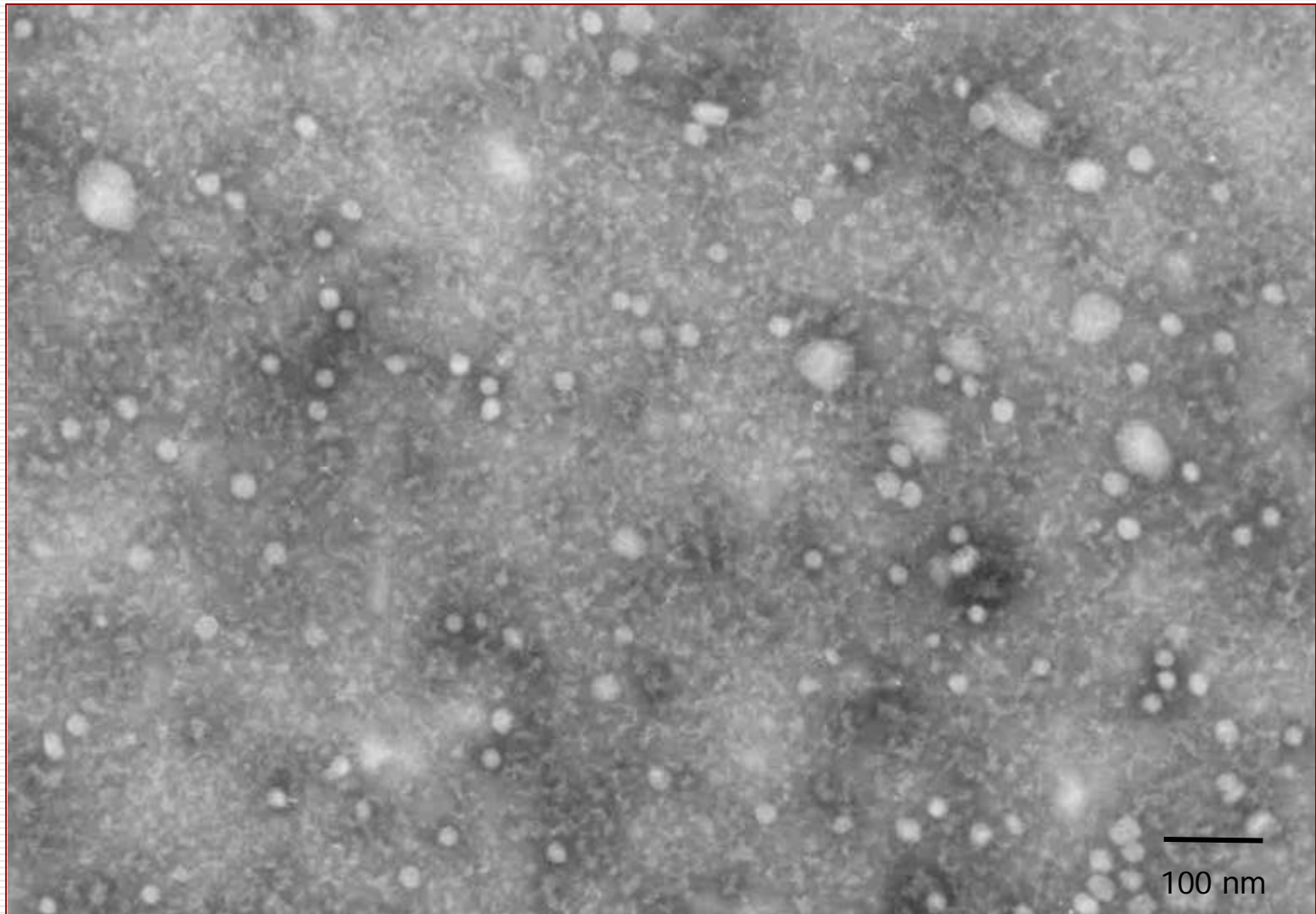
Deformed Wing Virus (DWV)



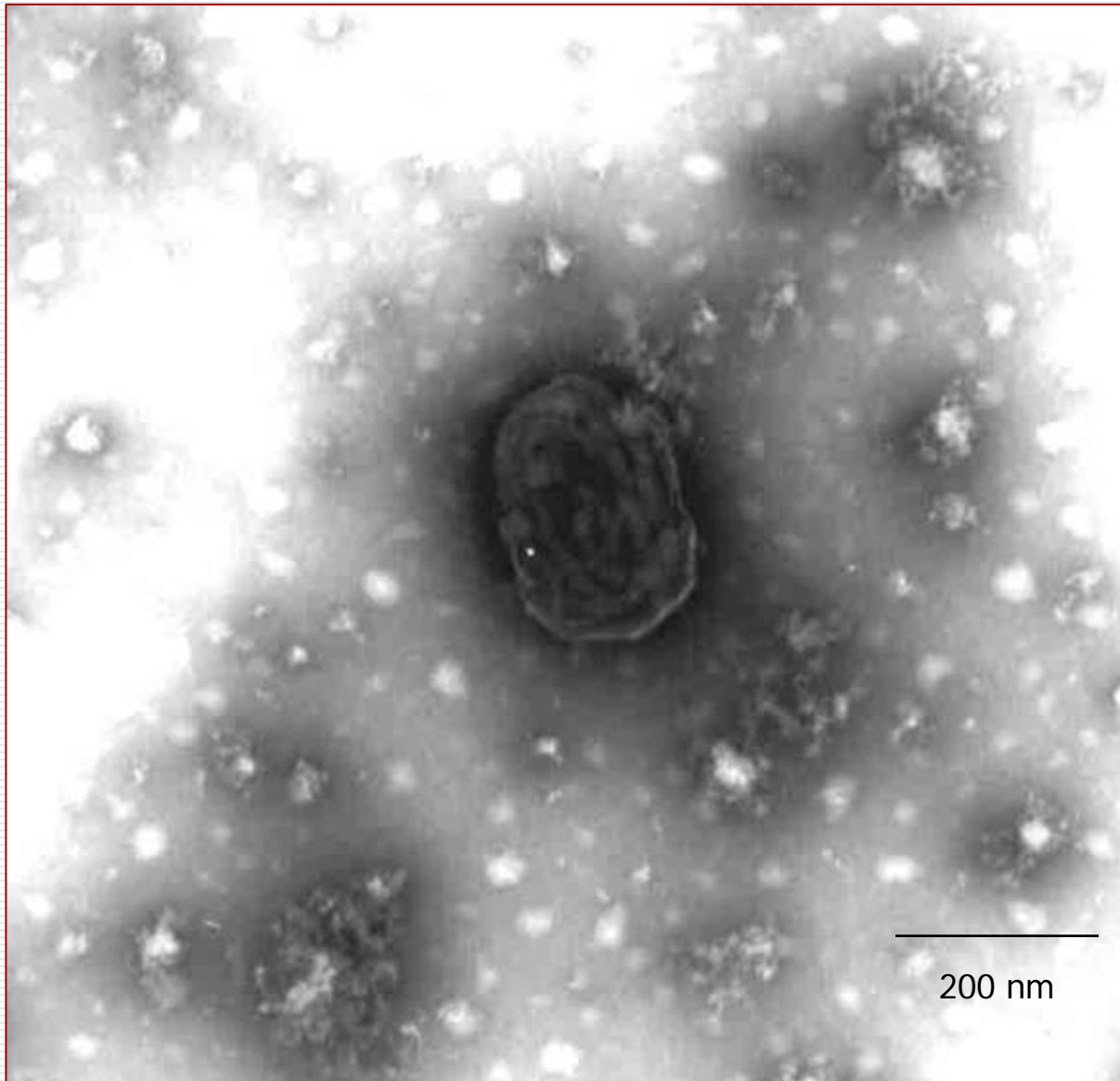
Sacbrood virus (SBV)



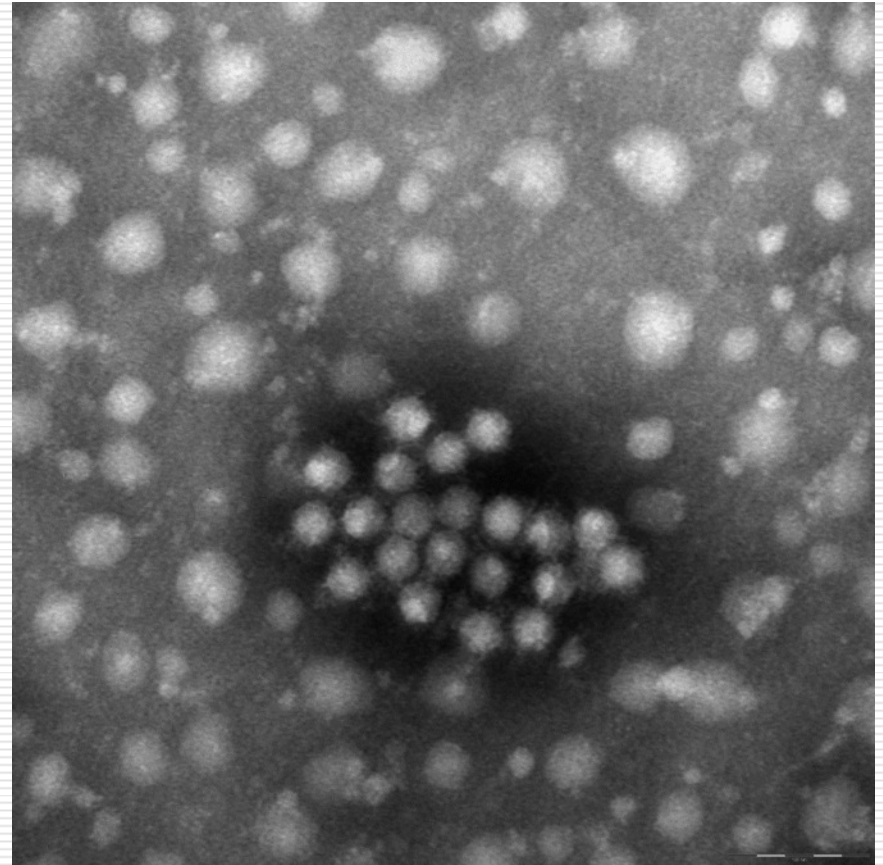
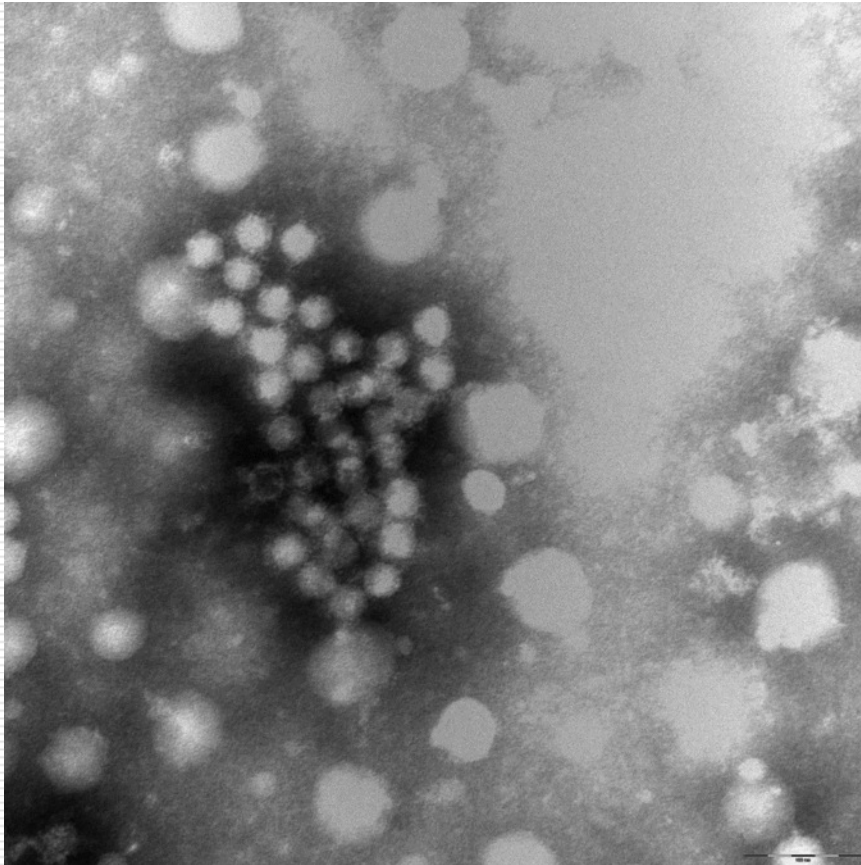
Cloudy Wing Virus (CWV)



Filamentous Virus (FV)



Picornavirus-like

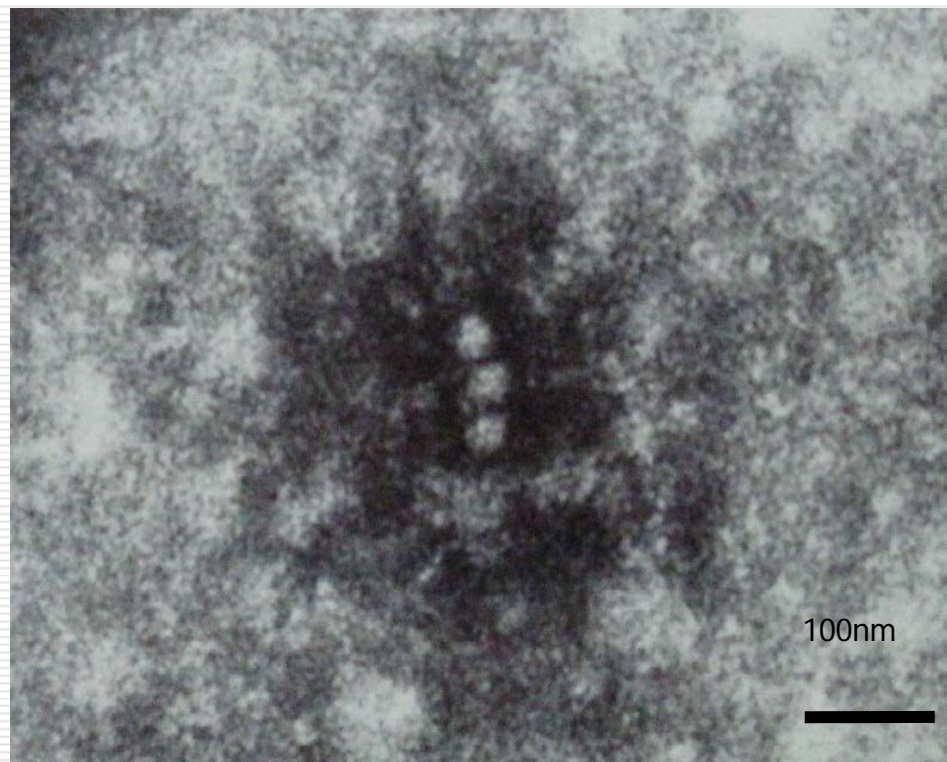


IEM, ELISA & RealTime PCR DWV: neg

ABPV Real Time PCR: neg

CBPV Real Time PCR: pos.

Israeli Acute Paralysis Virus (IAPV)



Journal of Apicultural Research 50(2): 176-177 (2011)
DOI 10.3896/IBRA.1.50.2.12

First detection of Israeli acute paralysis virus (IAPV) in Italy

Giovanni Formato¹, Alessandra Giacomelli¹, Ma'ayan Oliva², Lucie Aubin³, Eitan Glick⁴, Nitzan Paldi⁵, Giusy Cardeti¹, Antonella Cersini¹, Ilaria Maria Ciabatti¹, Massimo Palazzetti¹, Anna Granato¹ and Franco Mutinelli¹

¹Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Roma, Italy;

²Beeologics Inc., 11800 SW 77th Avenue, Miami, FL 33156, USA;

³ASL VT Sez 1 Servizio Veterinario AREA B, Viterbo, Italy;

⁴Istituto Zooprofilattico Sperimentale delle Venezie, NRL for beekeeping, Legnaro (PD), Italy;

Diagnosis of Poxviruses

- ✓ Rapid diagnosis in wild and domestic animals
 - Myxomatosis and Hare Fibromatosis
 - Avian poxvirus
- ✓ Differential diagnosis
 - Contagious Ecthyma (Orf) vs Bluetongue in sheep
 - Pustular Stomatitis vs Foot and Mouth Disease in Cattle
 - Swinepox vs skin bacterial and parasitic infections
- ✓ Zoonosis:
 - Cowpoxvirus (from cat and llama)
 - Bovine Pustular Stomatitis



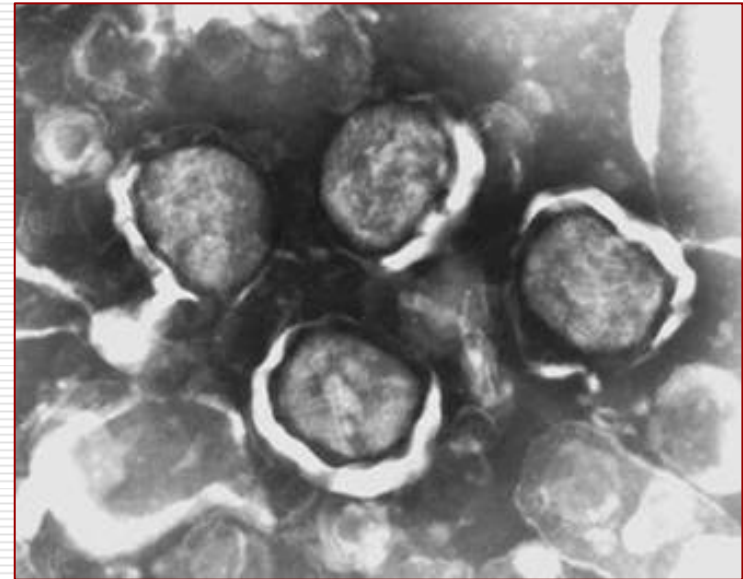
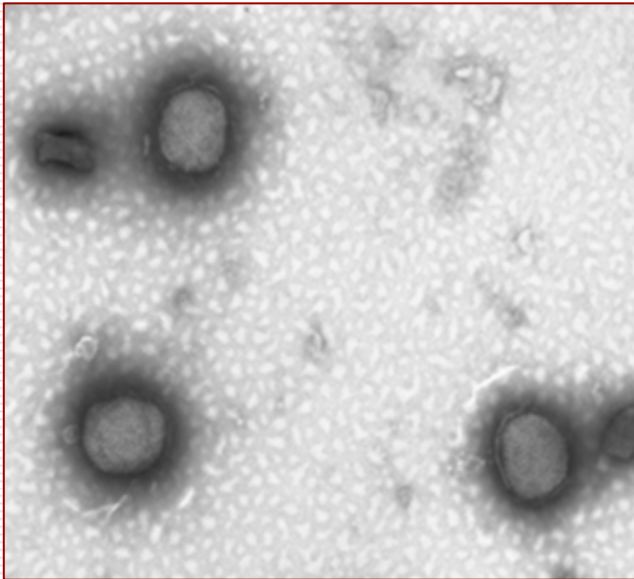
Rabbit Myxomatosis and Hare Fibromatosis



Endemic disease in wild and industrial rabbits

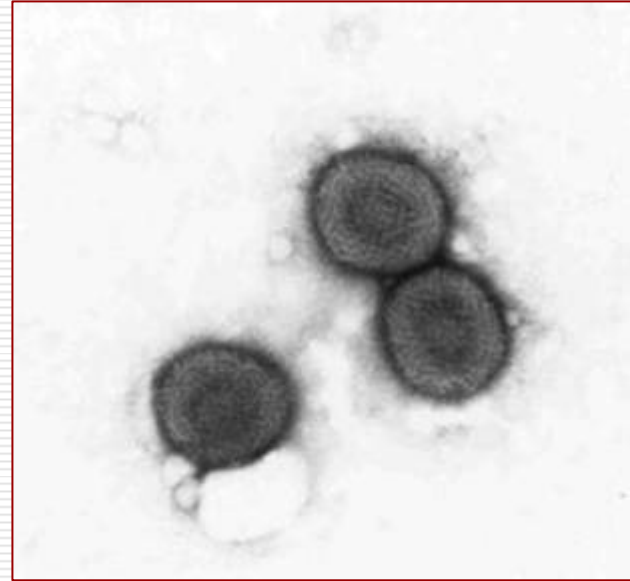
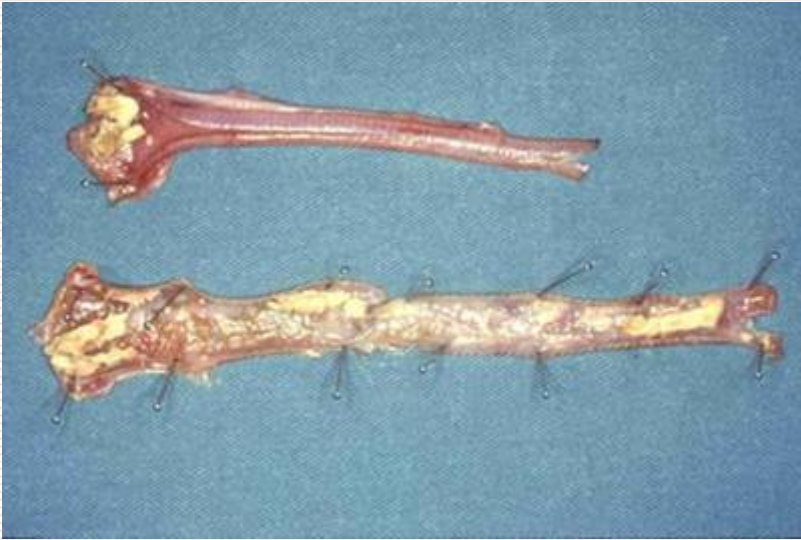


Re-emerging disease observed after 40 years

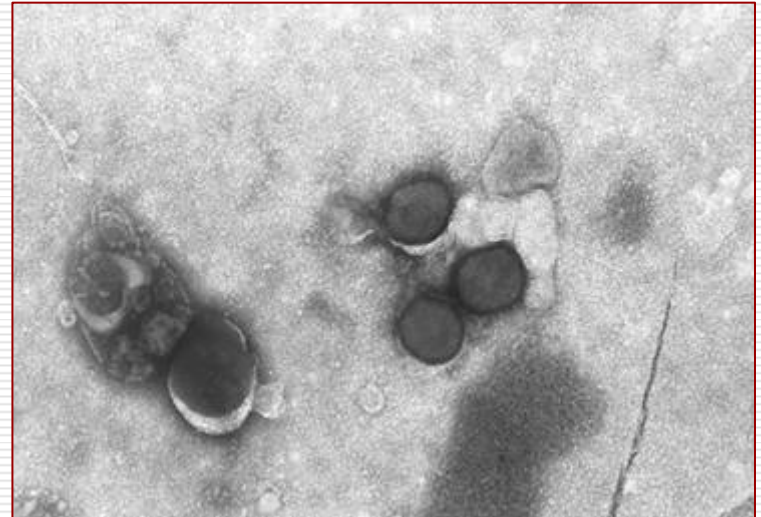


Avian Poxvirus

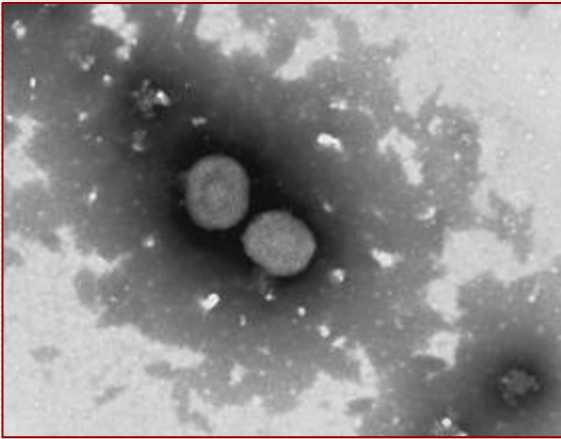
CHICKEN



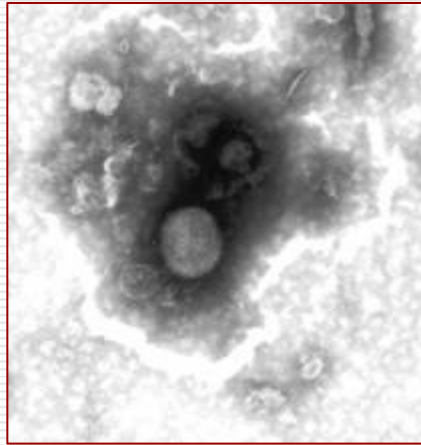
TURKEY



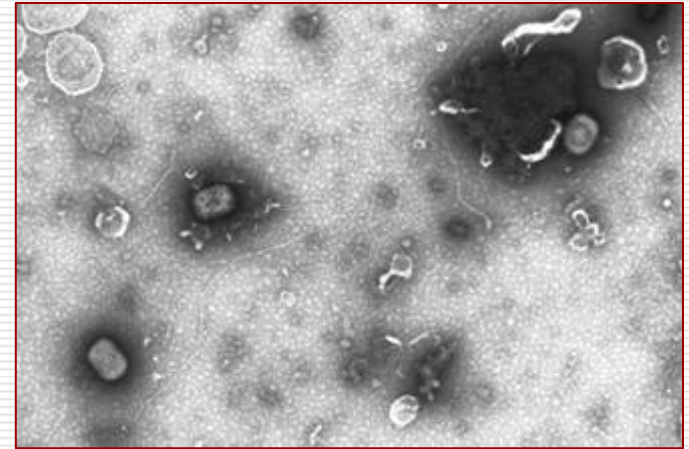
Avian Poxvirus



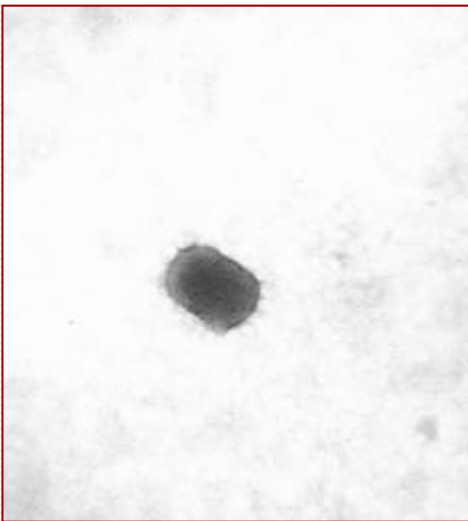
Owl



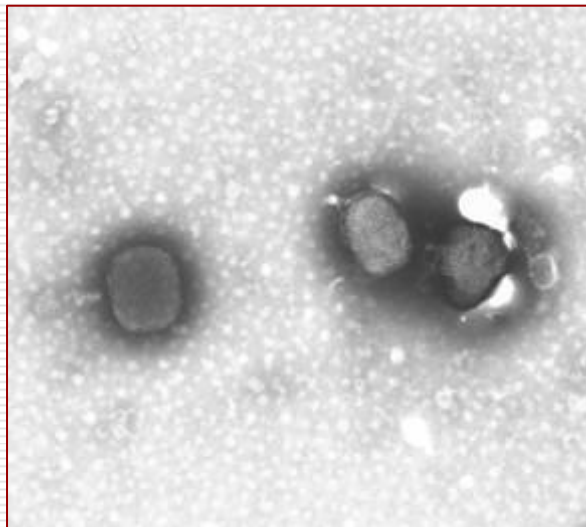
Grey Partridge



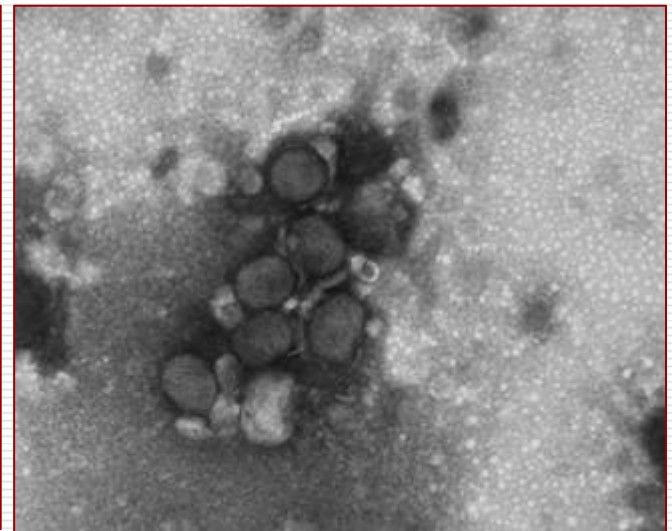
Ostrich



Sparrow

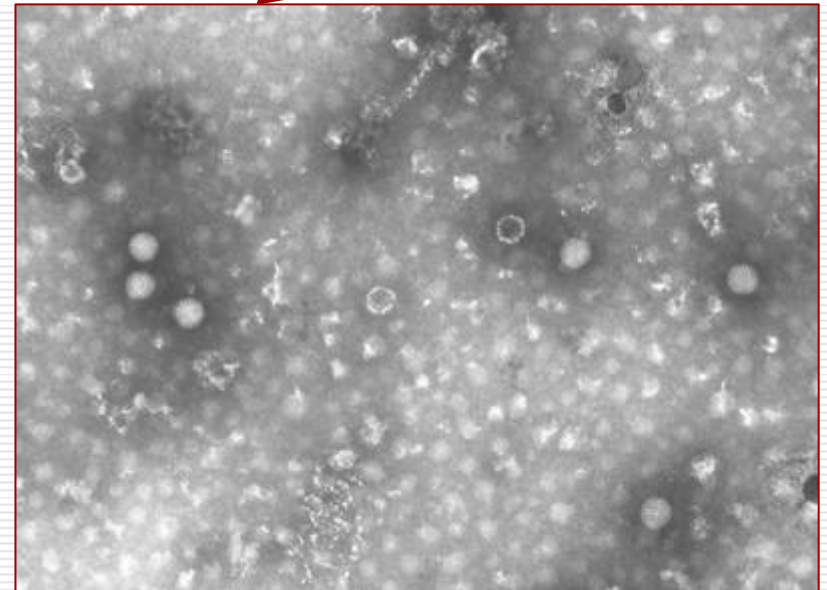
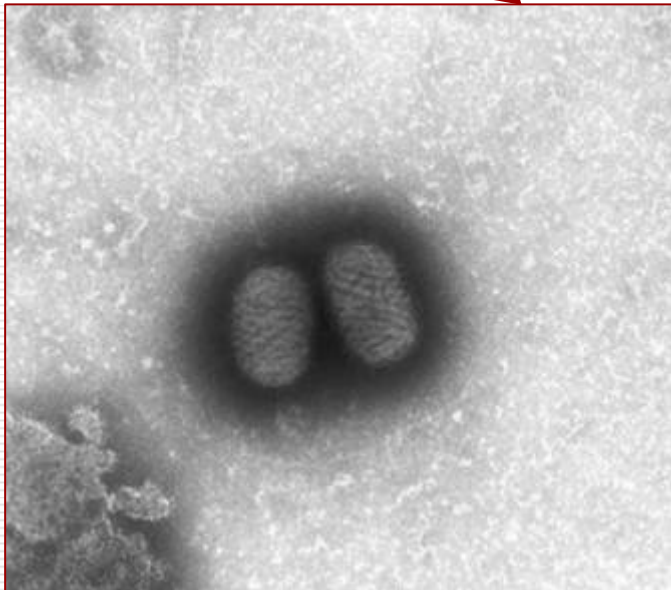


Canary

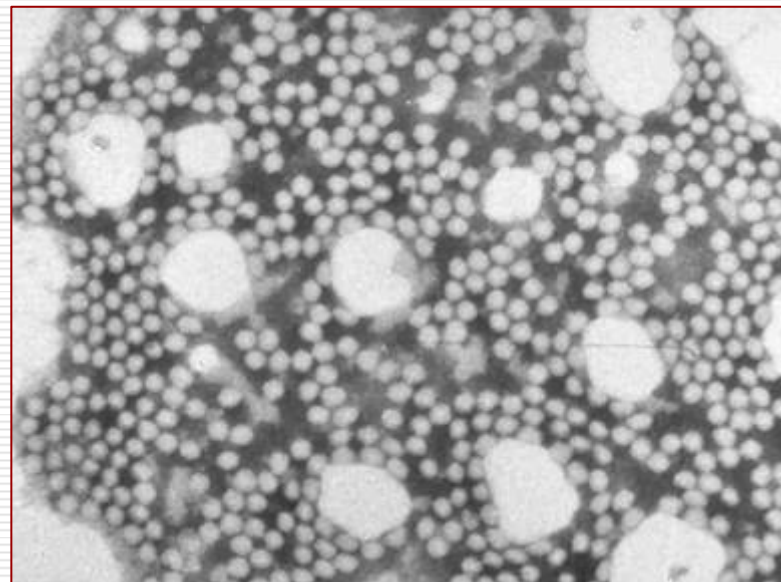
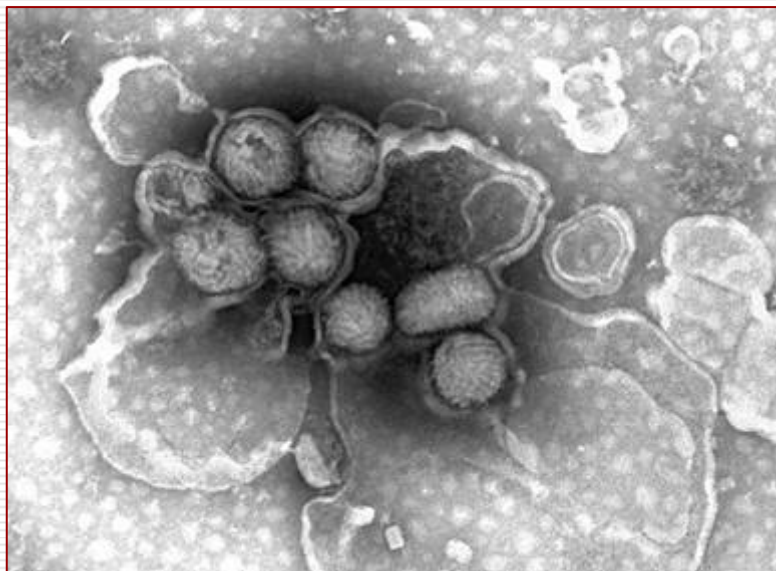


Hawk

Contagious ecthyma (ORF) vs BlueTongue in sheep



Pustular Stomatitis vs FMD in Cattle



Parapoxvirus infection in wild ruminants

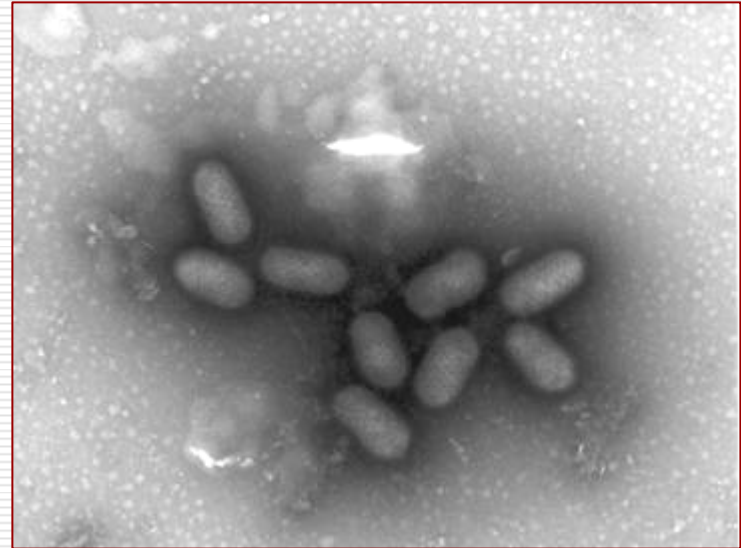
Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 17, No. 4, April 2011

DISPATCHES

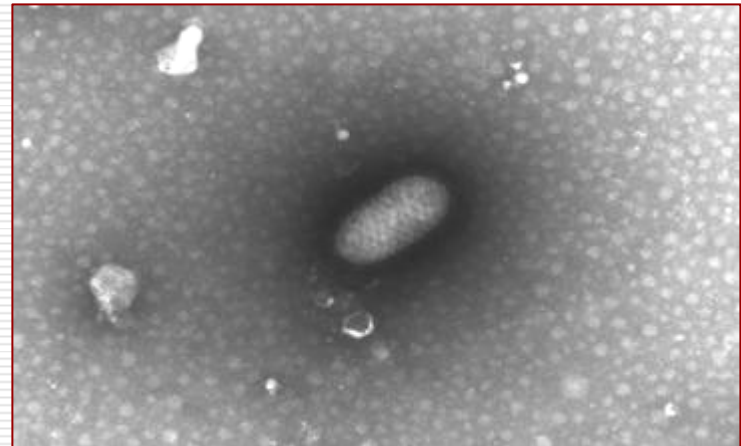
Parapoxvirus Infections of Red Deer, Italy

Alessandra Scagliarini,¹ Francesca Vaccari,
Filippo Turrini, Alessandro Bianchi,
Paolo Cordioli, and Antonio Lavazza

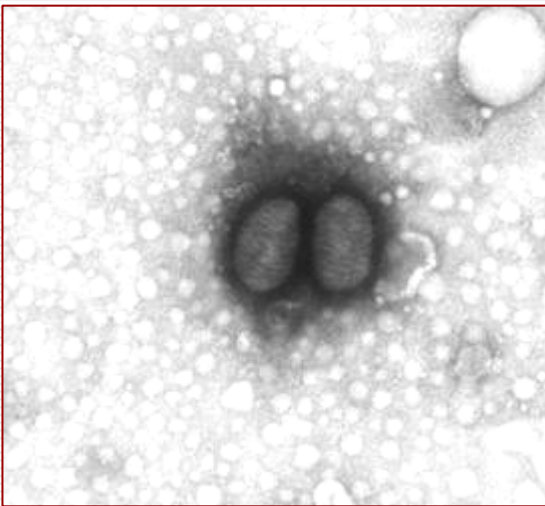
To characterize parapoxviruses causing severe disease in wild ruminants in Stelvio Park, Italy, we sequenced and compared the DNA of several isolates. Results demonstrated that the red deer isolates are closely related to the parapox of red deer in New Zealand virus.



Chamois

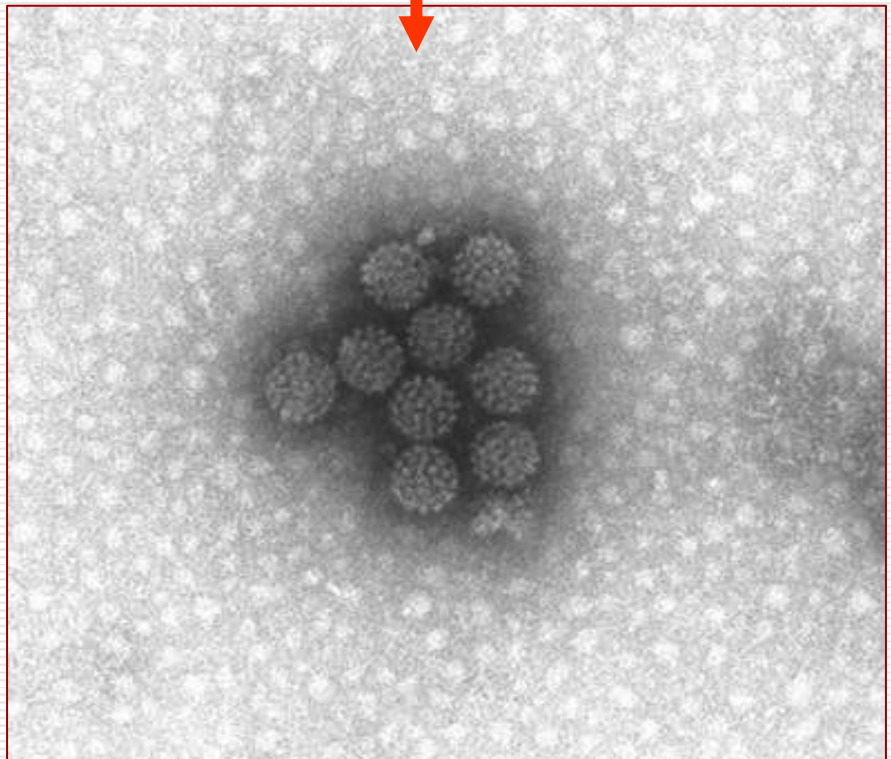
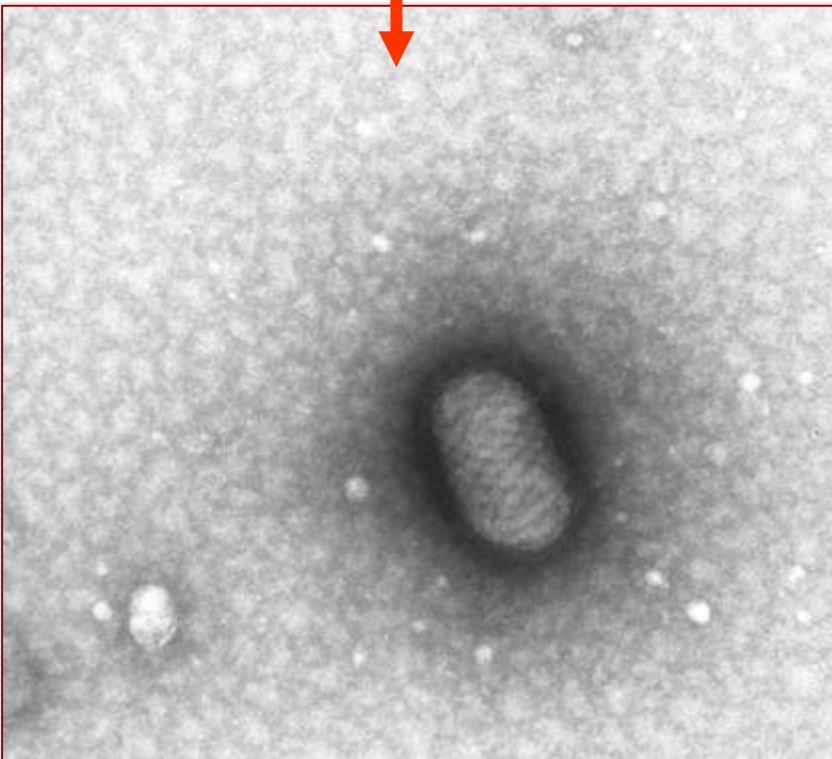


Alpine Ibex



Red deer

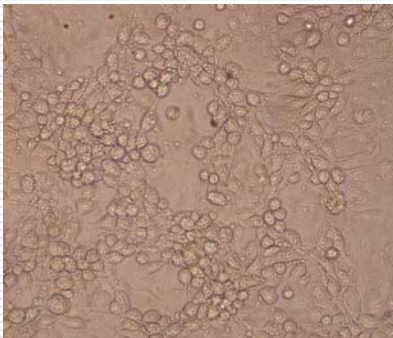
DD parapoxvirus vs papovavirus in wild ungulates



Swinepox virus



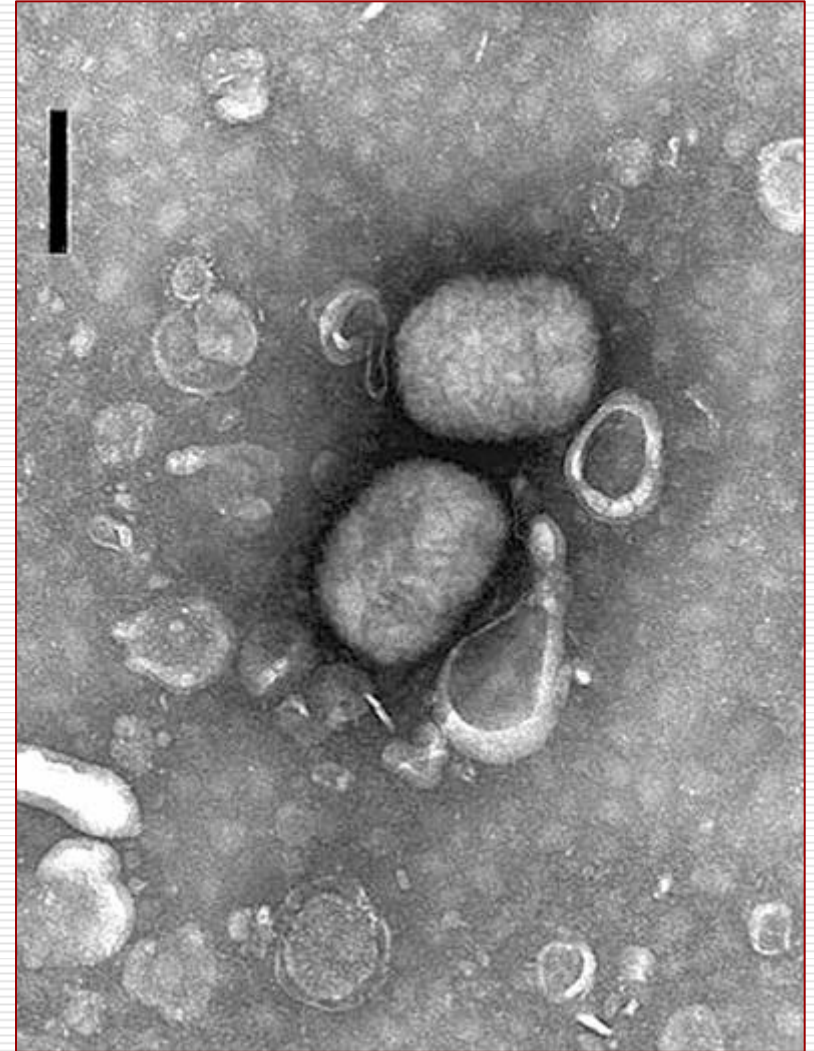
Observed directly from skin lesions
and from cell cultures with CPE



*CPE on PK15 cells
at 5 dd p.i.*

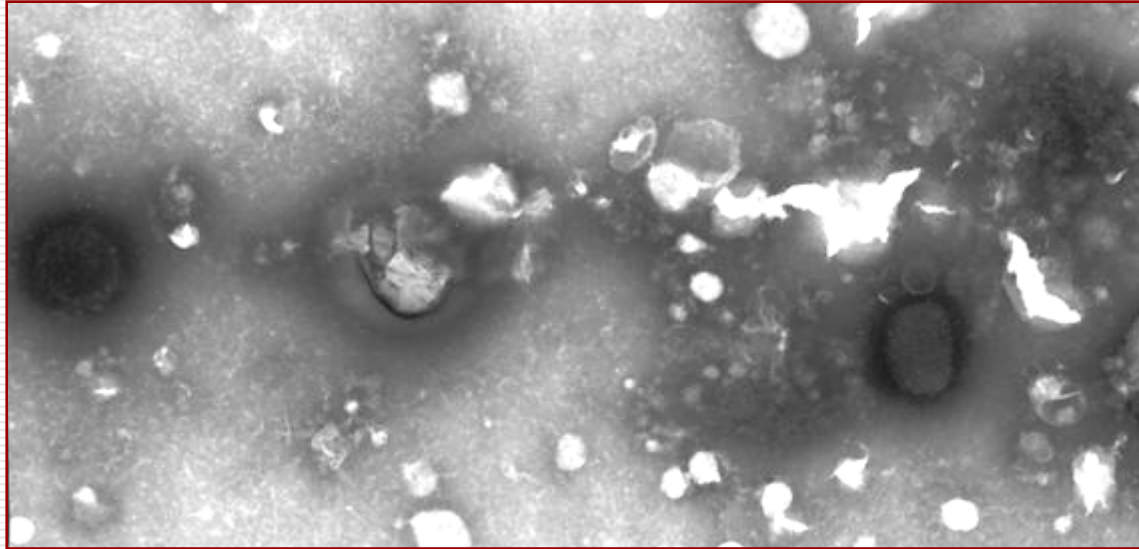


*CPE on primary porcine
kidney cells at 3 dd p.i.*

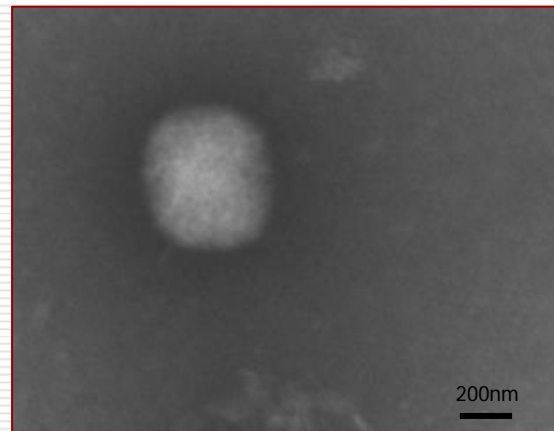


Cowpox virus

Cat



Llama



[Emerg Infect Dis.](#) 2011 Aug;17(8):1513-5. doi: 10.3201/eid1708.101912.

Cowpox virus in llama, Italy.

[Cardeti G](#), [Brozzi A](#), [Eleni C](#), [Polidi N](#), [D'Alterio G](#), [Carletti F](#), [Sciduna MT](#), [Castilletti C](#), [Capobianchi MR](#), [Di Caro A](#), [Autorino GL](#), [Amaddeo D](#).

Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Rome, Italy. giusy.cardeti@izs.it

ImmunoElectronMicroscopy (IEM)



More specific: morphological identification plus antigen specificity

More sensitive: identification of known and unknown viruses

- *with no peculiar morphology (pleomorphic, elusive virus)*
- *in low concentration (10^2 - 10^3 enrichment)*
- *in "dirty" samples (complex size)*

The "Airfuge" method + IEM

1) DILUTION of FAECAL SAMPLES

1:5 w/v in bi-distilled water

2) LOW SPEED CENTRIFUGATIONS

6000 and 10000 rpm for 30 min

3) IEM STEP

incubation with serum at 37°C for 1 hr

4) ULTRACENTRIFUGATION AIRFUGE BECKMAN

21 psi for 15 min

5) NEGATIVE STAINING

2% NaPt, pH 6.8 for 1.5 min


6) OBSERVATION

TEM - 80 kV at 19000-34000x



Type of sera used for IEM

- × Hyperimmune serum
 - of the same species
 - of heterologous species
- × Monoclonal antibodies
- × Convalescent serum



Why ?

Emerging o re-emerging virus as etiological agents of undiagnosed clinical outbreaks

Possible failure of alternative diagnostic methods due to

1. *no immunological reagents*
2. *no primers available*
3. *clinical suspect not indicative*

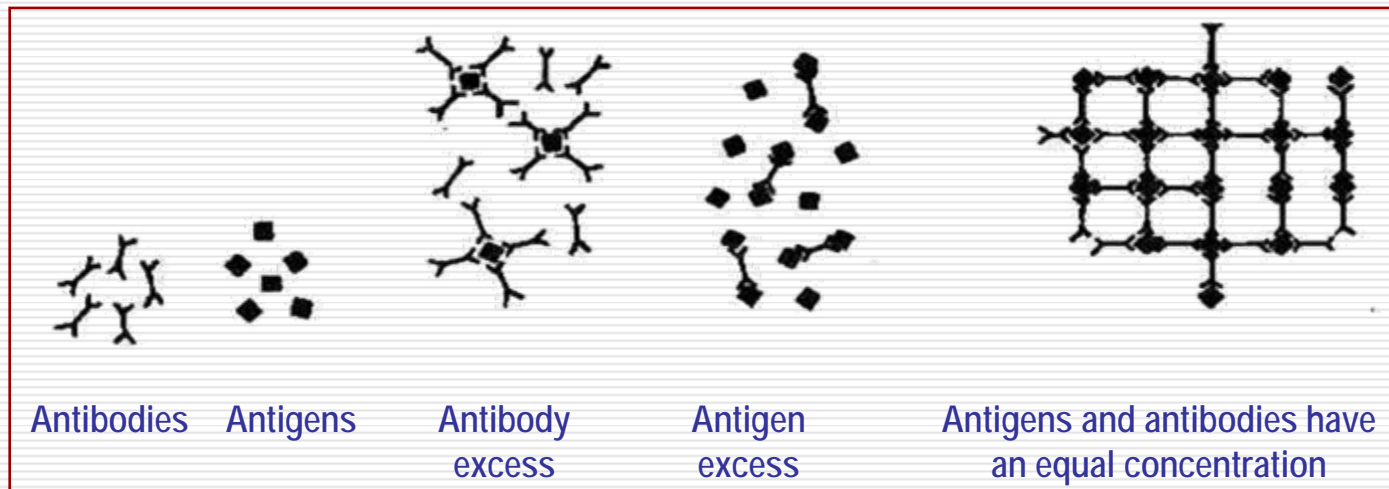
Preparation of the convalescent serum

- ✖ Pooling of 2-10 sera taken from convalescent animals 15-20 dd after clinical signs
- ✖ Centrifugation at 10000 rpm for 30 min.
- ✖ Inactivation by heating at 56°C for 30 min.
- ✖ Filtering through 22 µm filters
- ✖ Examination by EM: free from viral particles, lipid and protein debris
- ✖ Testing the sample with serial dilutions
 - ✖ *Initially 1:2, 1:20, 1:200 and 1:1000.....*
 - ✖ *....and, according to the results, 1:10, 1:50, 1:100, 1:500 etc.*

Optimal dilution of the serum and controls

The **dilution** is **optimal** when:

- ✗ *The immunoaggregates are of adequate size*
- ✗ *The halo of antibodies around virions is not so thick*
- ✗ *The morphology of virions is not altered*



Controls (*spontaneous aggregations of virions ?*)

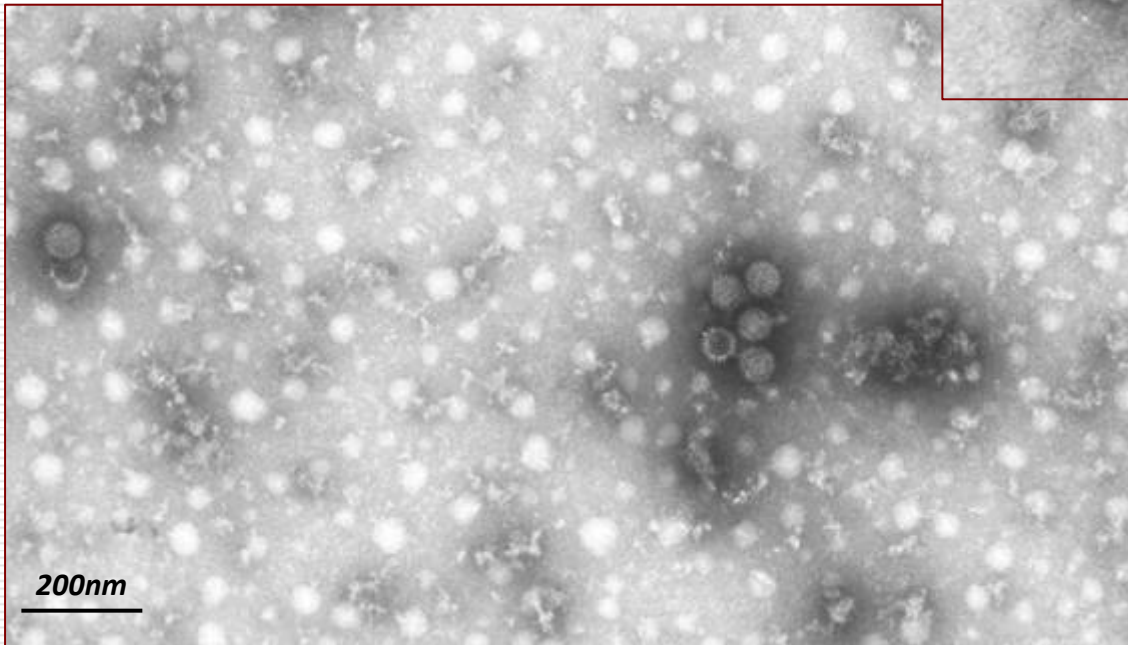
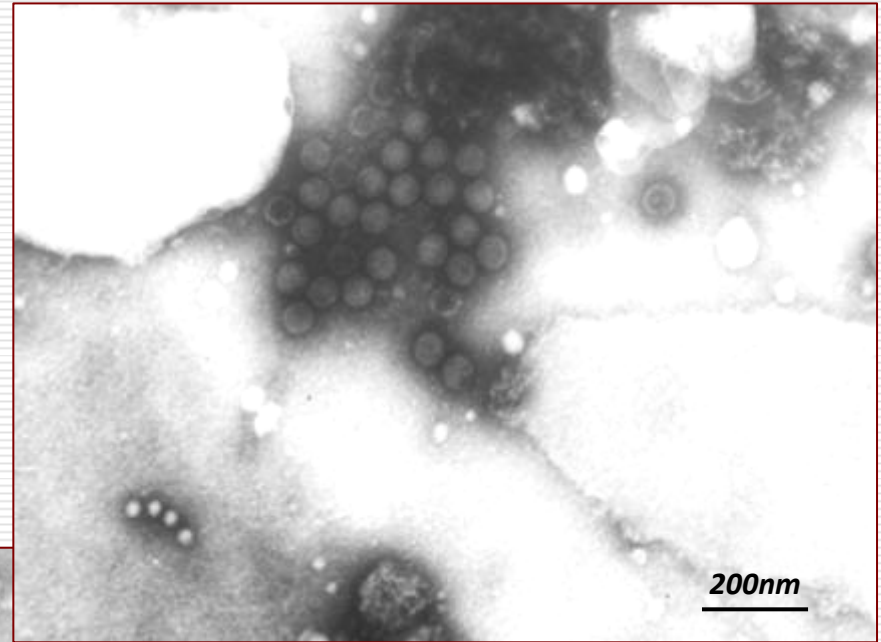
- ✗ *Sample with no serum*
- ✗ *Incubation with a pre-serum (if available)*
- ✗ *Incubation with another serum*

Examples of using convalescent sera to detect “new” and emerging viruses by IEM

- Porcine Rotavirus (PRoV)
- Porcine Epidemic Diarrhea Virus (PEDV)
- Porcine Circovirus (PCV-2)
- Porcine Torovirus (PToV)
- Turkey Rota- and Astrovirus
- Pheasant Parvovirus-like
- Lagovirus (RHDV and EBHSV)

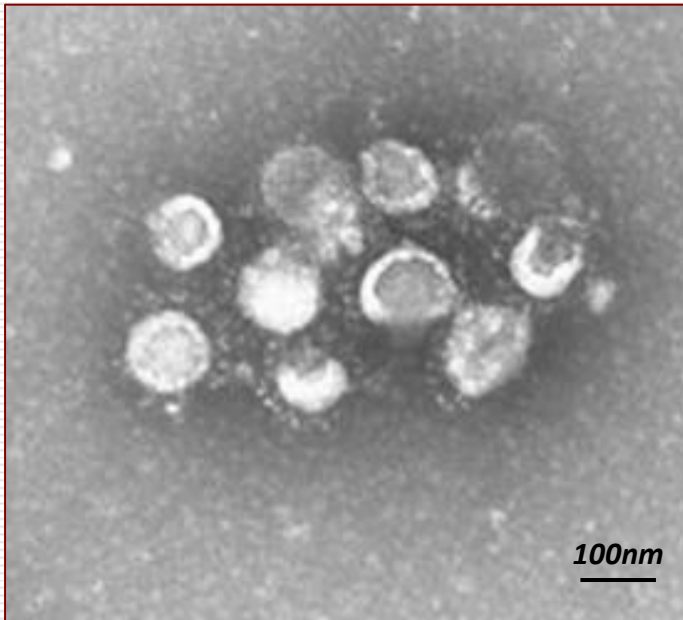
Porcine Rotavirus

- ✓ Primary agents of enteritis
- ✓ Steady incidence
- ✓ Often associated to other viruses
- ✓ Convalescent serum: many strains belong to non-A types



Porcine Epidemic Diarrhea Coronavirus (PEDV)

- One of the four porcine coronavirus known (group 1)
- Clinical signs not typical: diarrhoea, vomit
- High morbidity, low mortality
- Constantly found and endemic in Italy
- Several outbreaks reported on 2005-2006

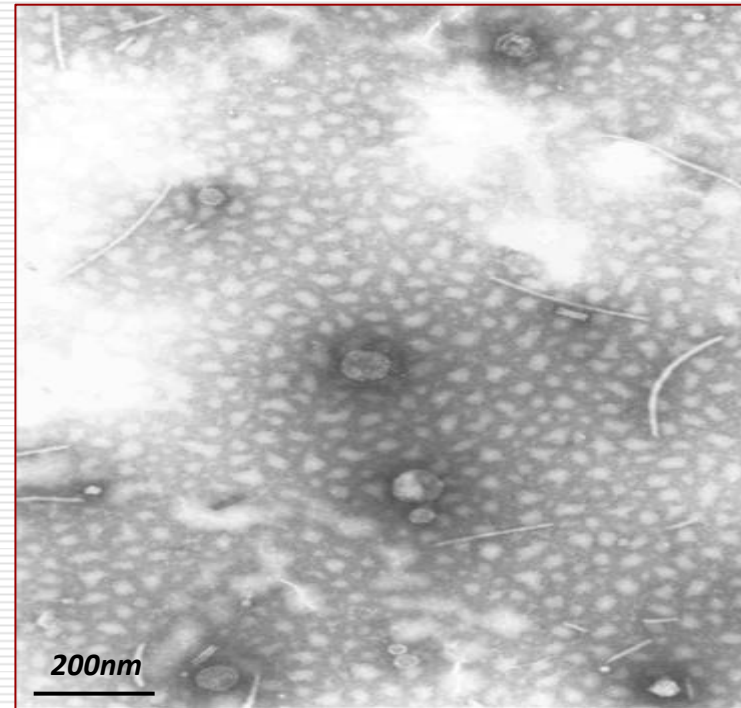
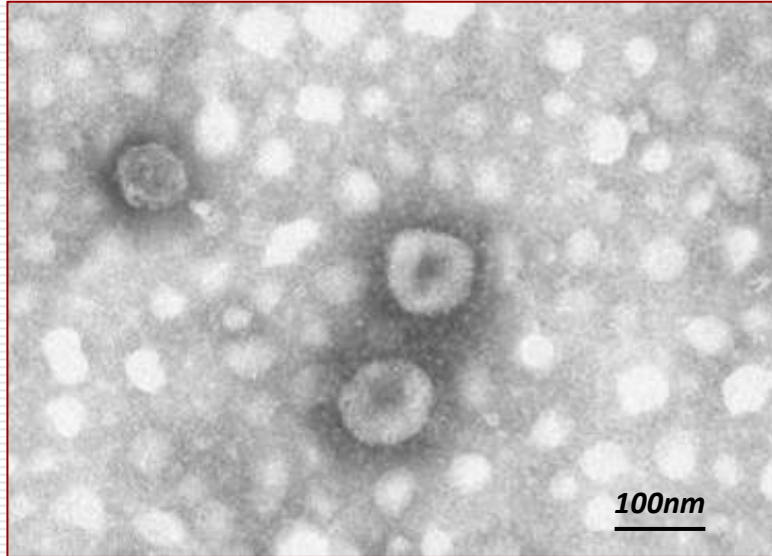
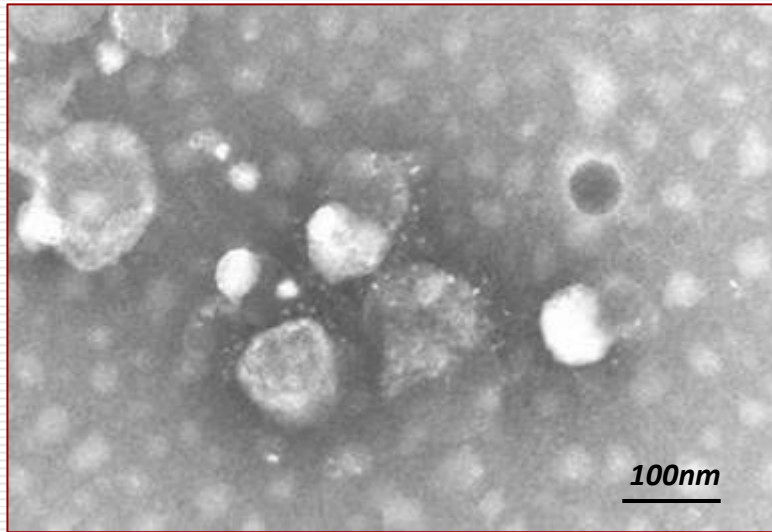


Veterinary Record (2008)
162, 307-310

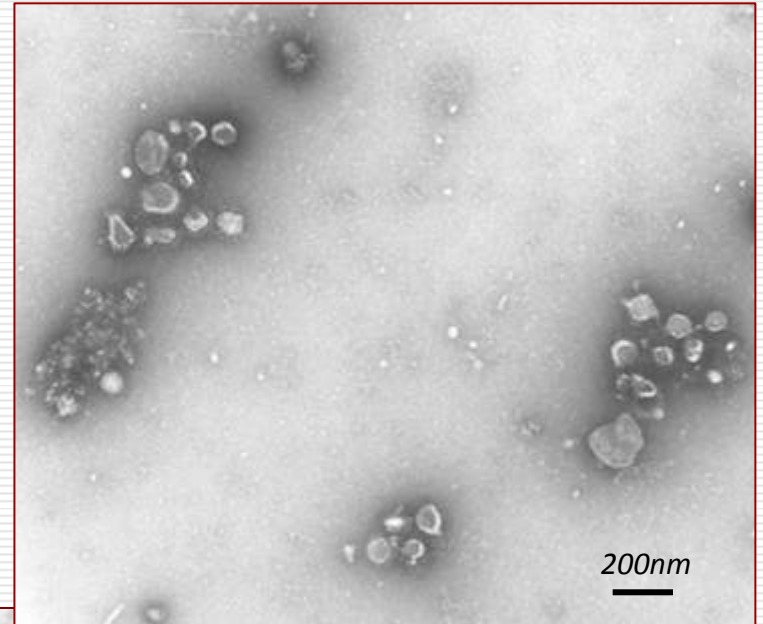
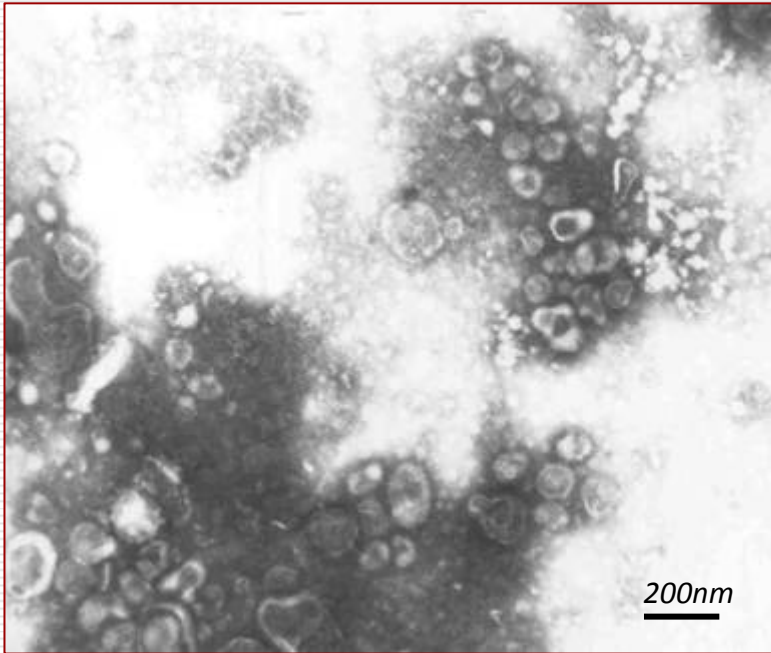
Epidemic of diarrhoea caused by porcine epidemic diarrhoea virus in Italy

P. MARTELLI, A. LAVAZZA, A. D. NIGRELLI, G. MERIALDI, L. G. ALBORALI, M. B. PENZAERT

PED coronavirus: single particles

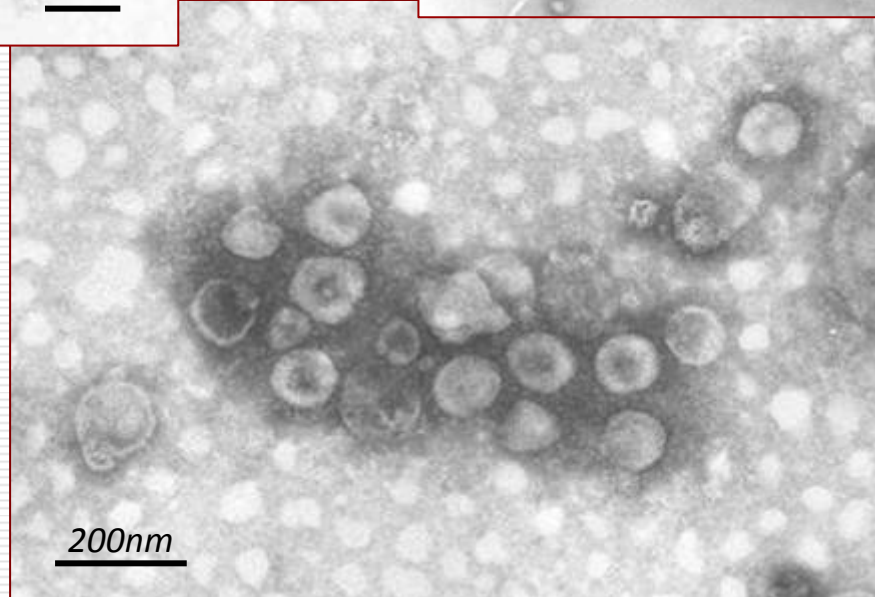


PED Coronavirus: aggregated groups (IEM)



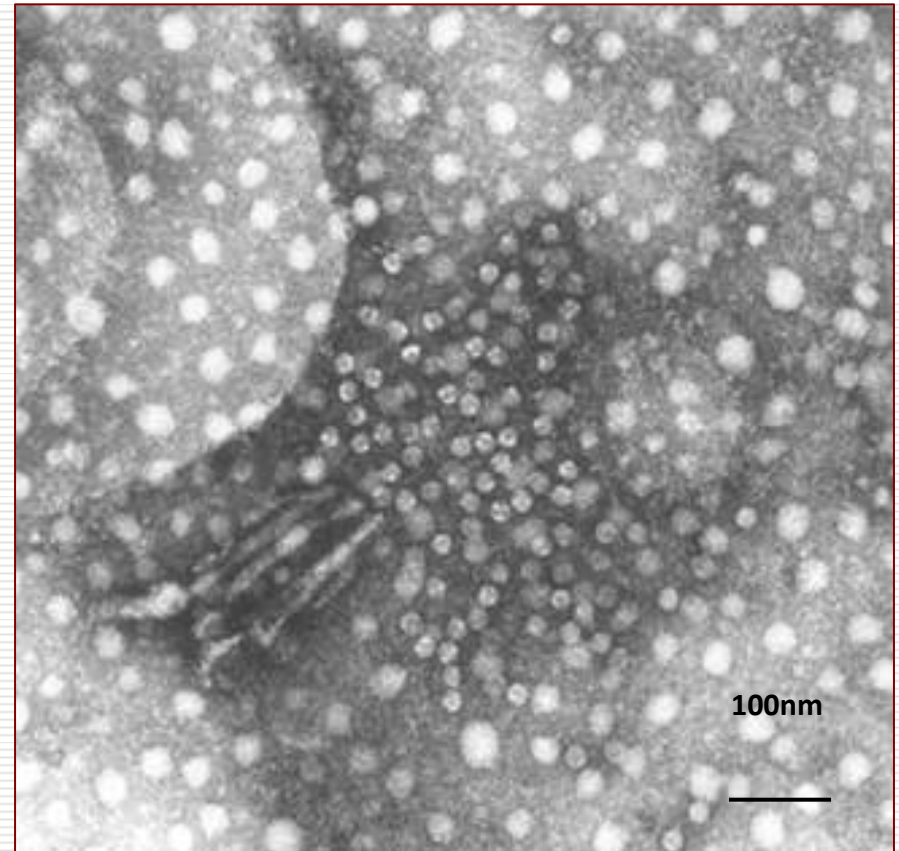
Convalescent serum:

PEDV is difficult to diagnose: no isolation *in vitro*, few reagents and established methods



Pig Circovirus (PCV-2)

- Circoviridae family
- ssDNA virus
- Not enveloped
- 17 nm in diameter
- PCV-1, not pathogenic, isolated on 1974 as contaminant of PK15
- PCV-2 isolated on 1991 and now associated with the Post-weaning Multisystemic Wasting Syndrome (PMWS)

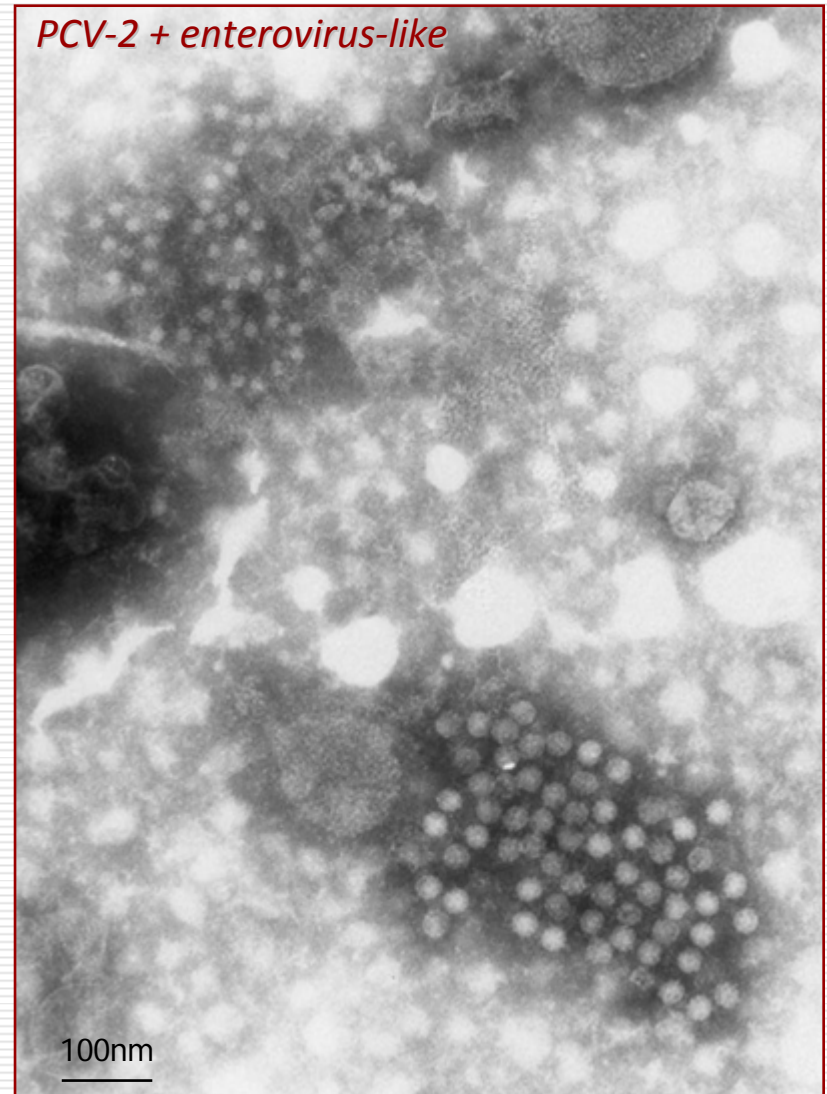
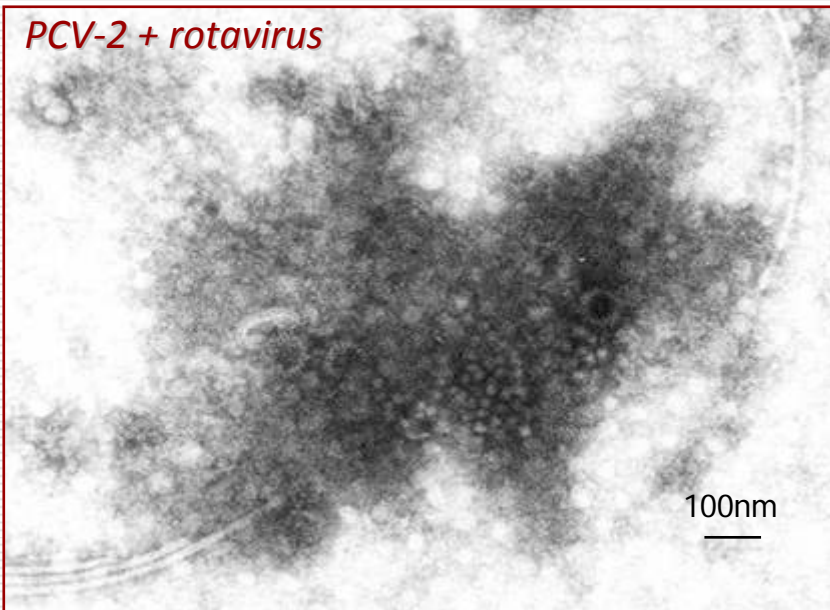


100nm



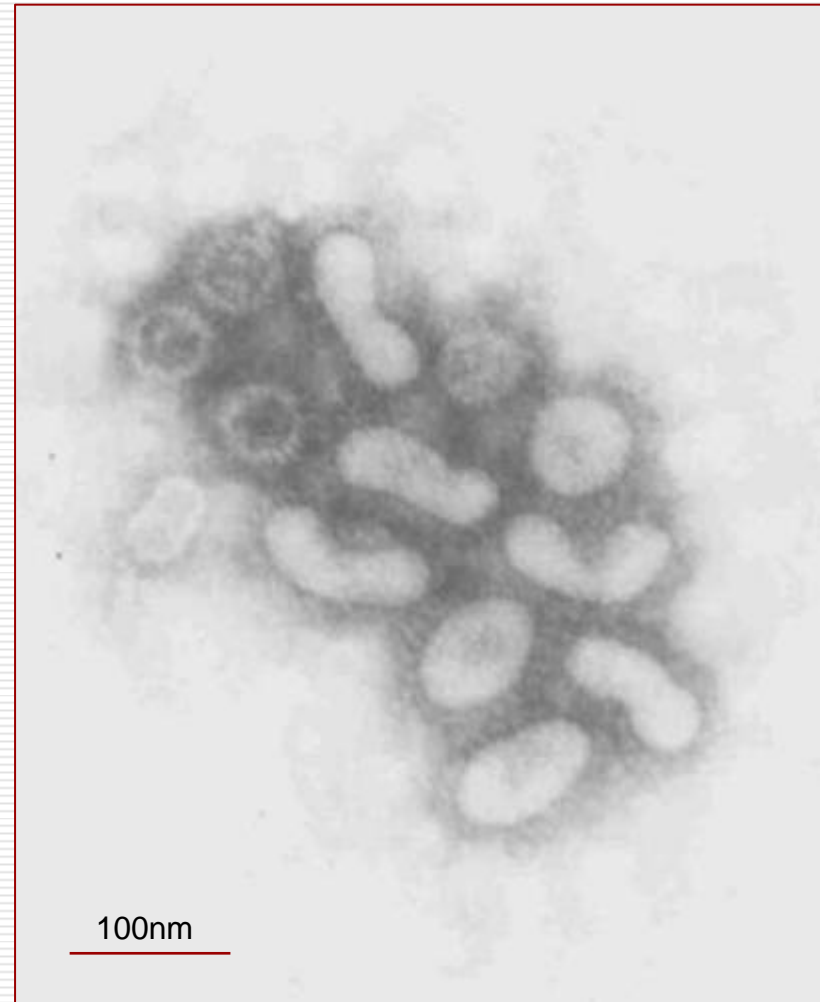
Pig Circovirus (PCV-2)

- ✓ Convalescent serum: PCV-2 frequently detected in piglets clinically affected by PMWS
- ✓ PCV-2 is often associated with other agents (immuno-suppressive effect)



Pig Torovirus (PToV)

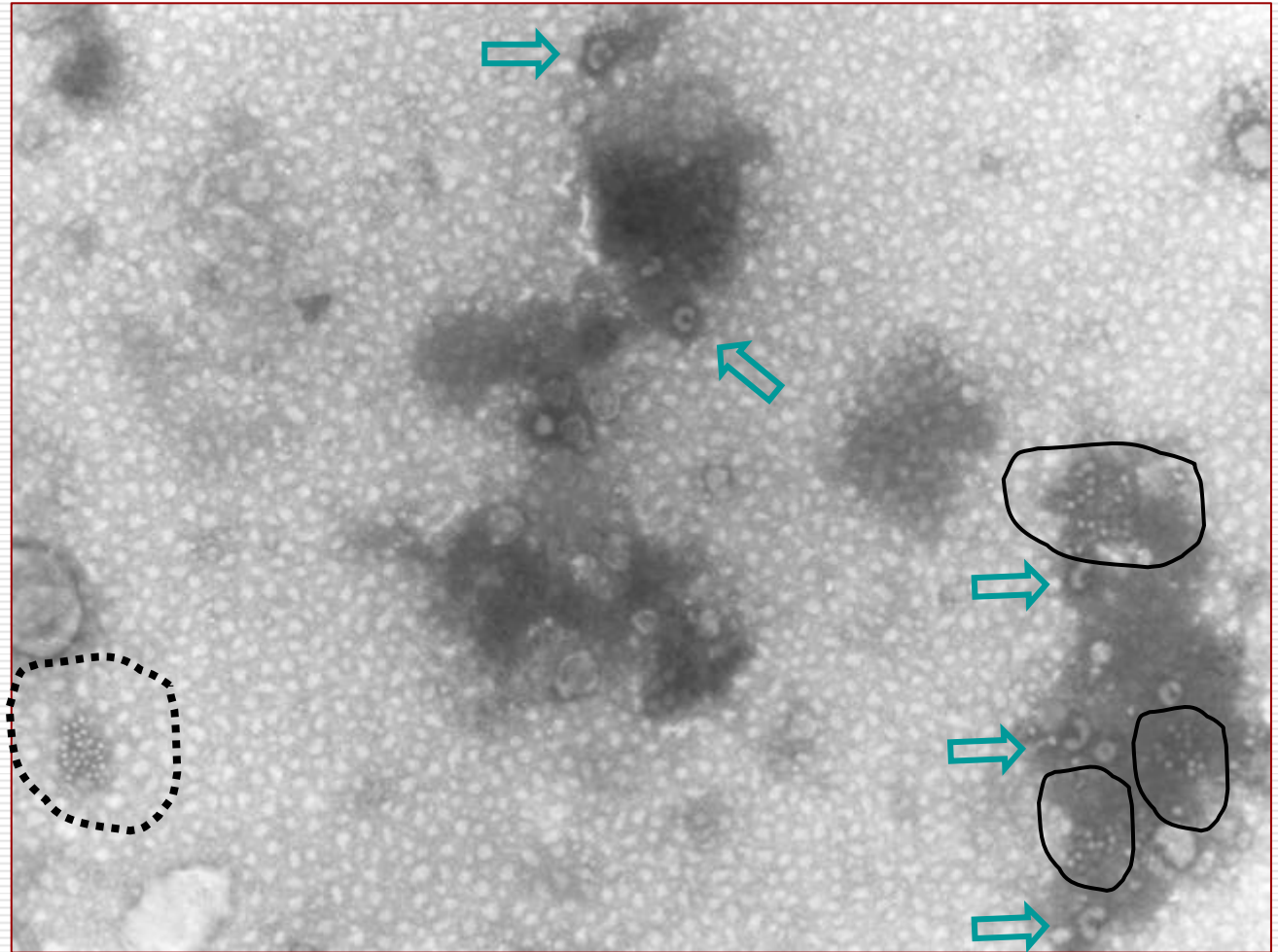
- Order *Nidovirales*
- Pos-stranded RNA viruses
- Discoidal, kidney- and rod-shaped; tubular nucleocapsid; envelope with 7-9 nm spikes
- Asymptomatic enteric infections in **swine**
- Serious, at times fatal, diarrheal disease in **cattle** (Breda virus-BRV).
- Gastroenteritis in **humans**



Pig Torovirus (PToV)

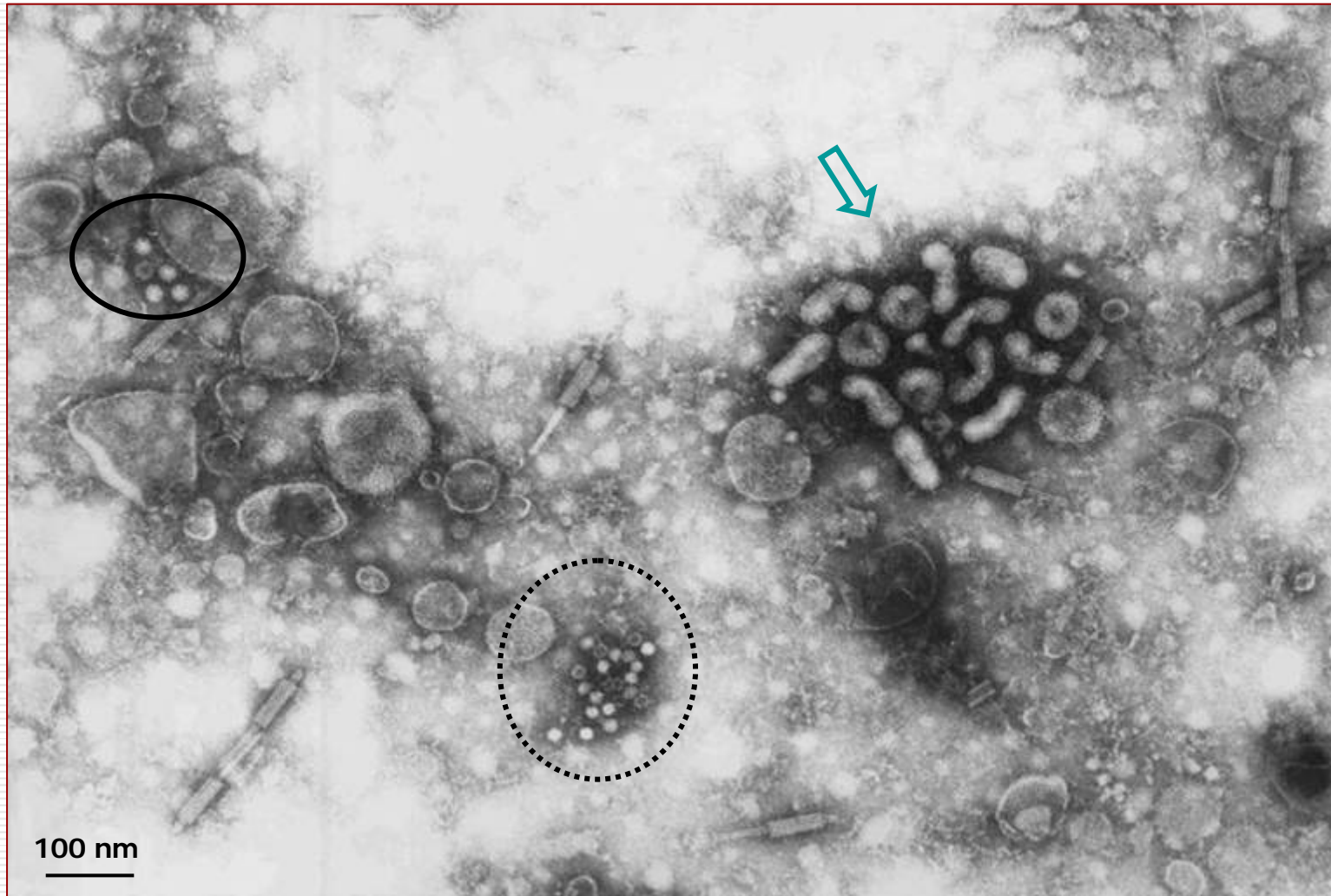
torovirus (\Rightarrow) + enterovirus-like (—) + PCV.2-like (···)

Convalescent serum: after the first case on 1990, four other cases detected between 1999-2002



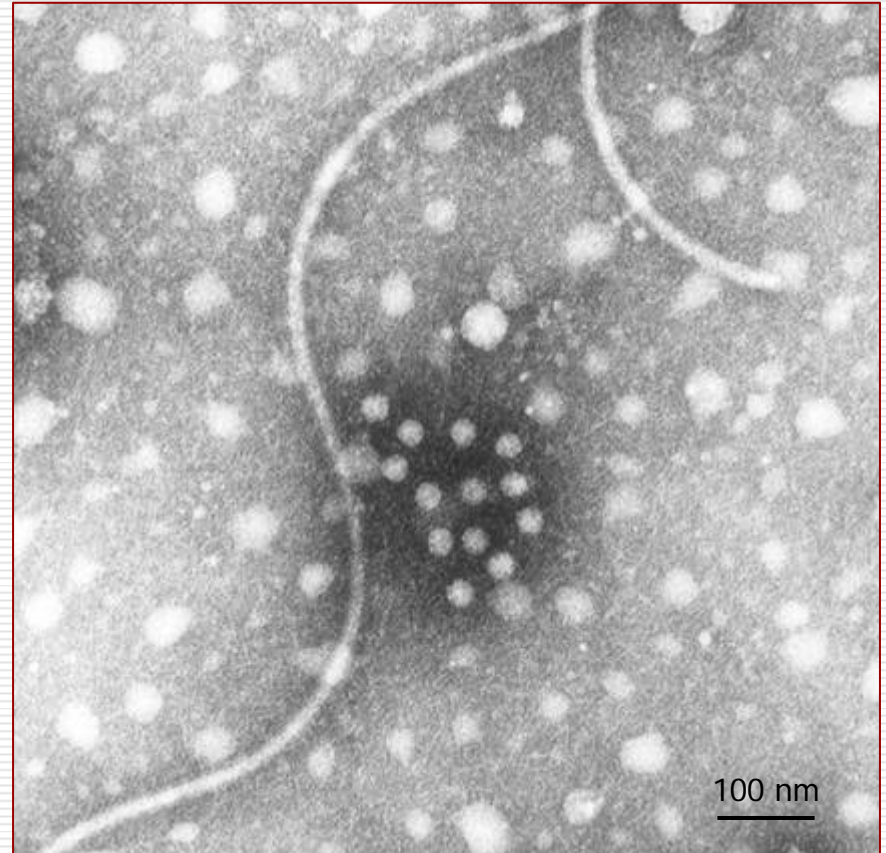
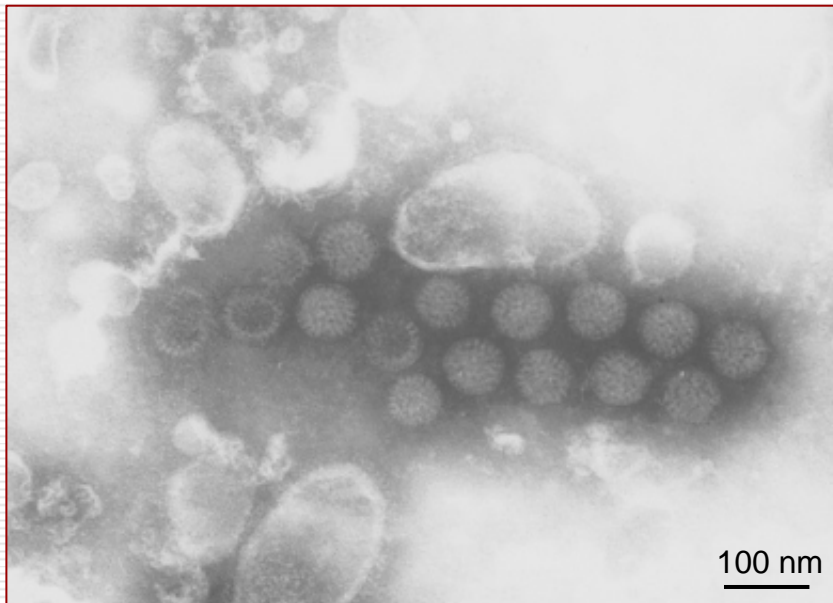
Pig Torovirus (PToV)

torovirus (\Rightarrow) + enterovirus-like (—) + PCV.2-like (···)



Turkey Rotavirus and Astrovirus

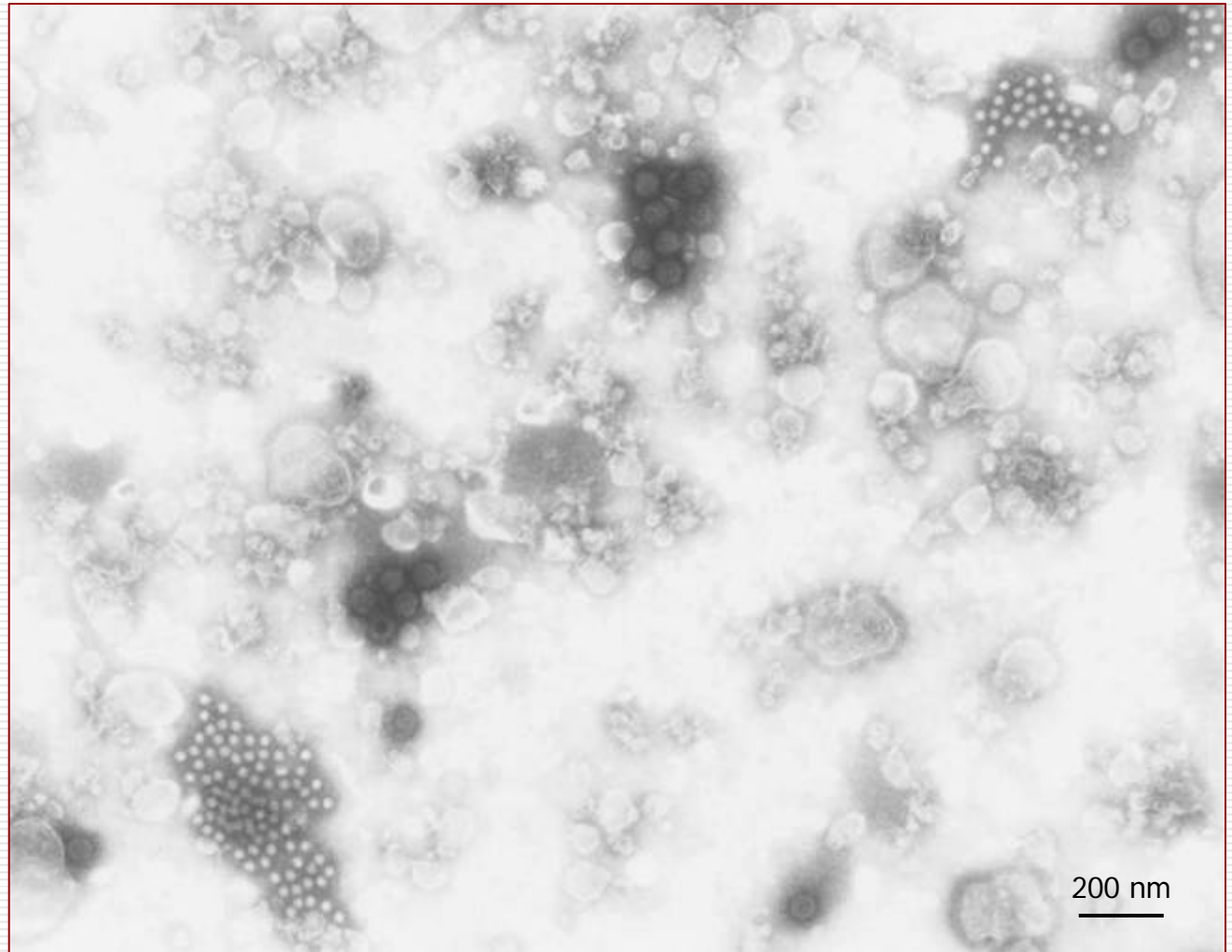
- Primary agents of enteritis in young turkeys (7-21 dd old)
- Often detected in association (synergic effect)



Turkey Rotavirus and Astrovirus

Convalescent
serum:

simultaneous
detection of
no-cultivable
viruses in
association



200 nm

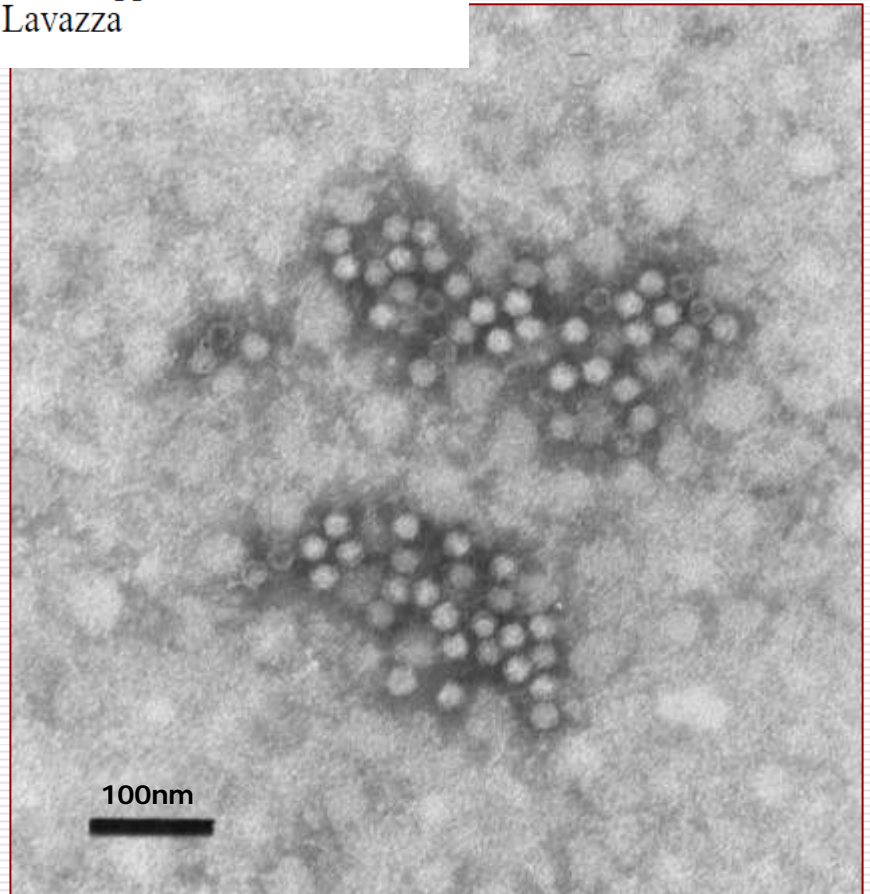
Pheasant Hepatitis

J Vet Diagn Invest 8:108-112 (1996)

Identification of parvovirus-like particles associated with three outbreaks of mortality in young pheasants (*Phasianus colchicus*)

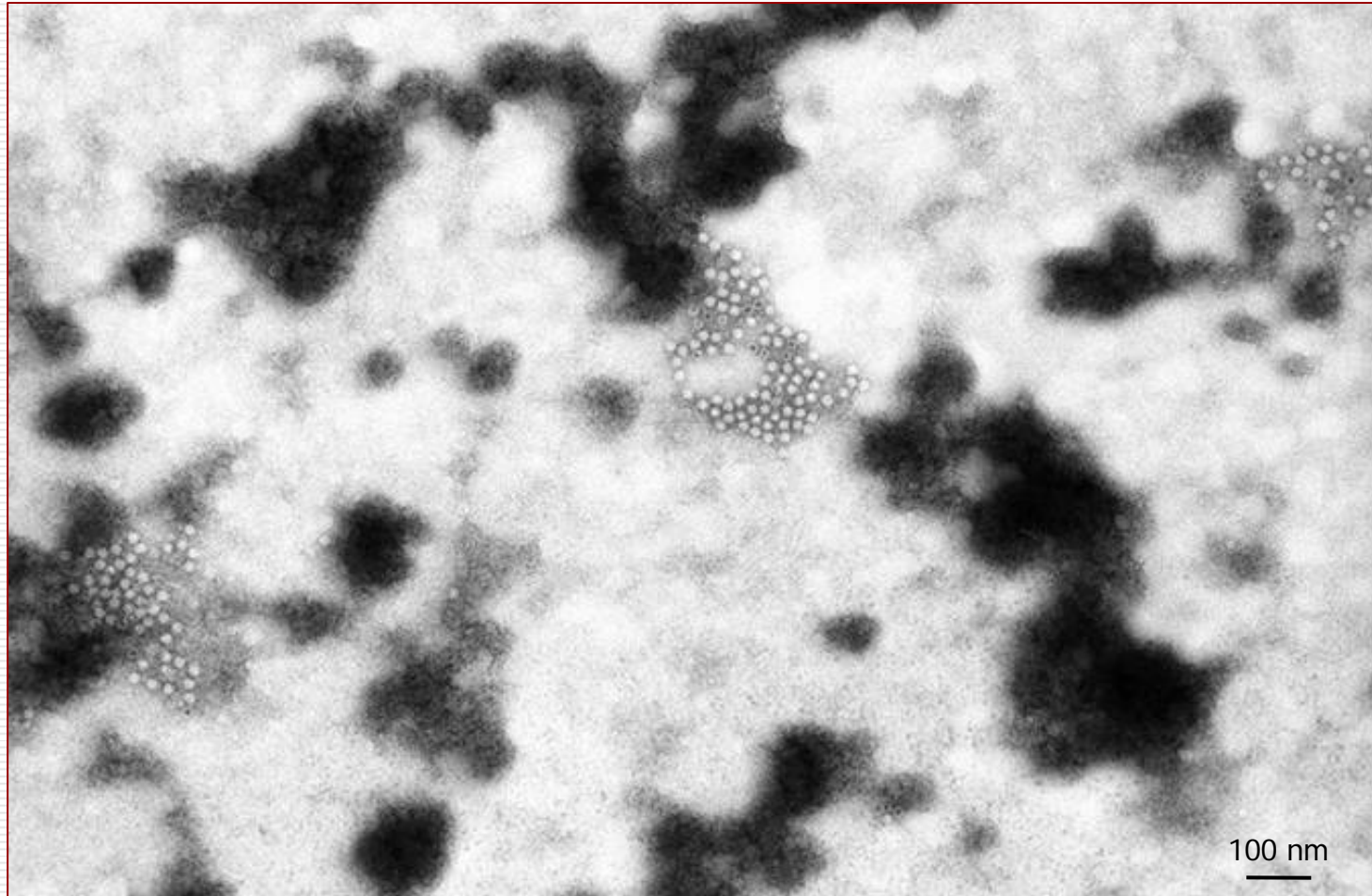
Daniela Gelmetti, Massimo Fabbi, Giuseppe Sironi,
Guido Grilli, Antonio Lavazza

- ✖ Observed in Italy on 1996
- ✖ New disease of young pheasants (15-25 dd)
- ✖ Anorexia, dullness, diarrhoea, loss of weight
- ✖ Diffused haemorrhages, enlarged livers
- ✖ Catarrhal enteritis
- ✖ 15-60% mortality



Pheasant Hepatitis

Convalescent serum: primary identification of 18-21 nm parvovirus-like virions in the liver of young pheasant



Lagovirus (RHDV and EBHSV)

RHD

Rabbit Haemorrhagic Disease



Domestic and wild rabbit
(*Oryctolagus cuniculus*)

EBHS

European Brown Hare Syndrome



European brown hare
(*Lepus europaeus* P.)

CALICIVIRUS (RHDV)

CALICIVIRUS (EBHSV)

Antigenically related but different viruses, no cross-infection

Firstly reported on 1984 in China
and on 1986 in Europe.

World-wide diffused and described
in over 40 countries

Morbidity 90-100%

Mortality 40-90%

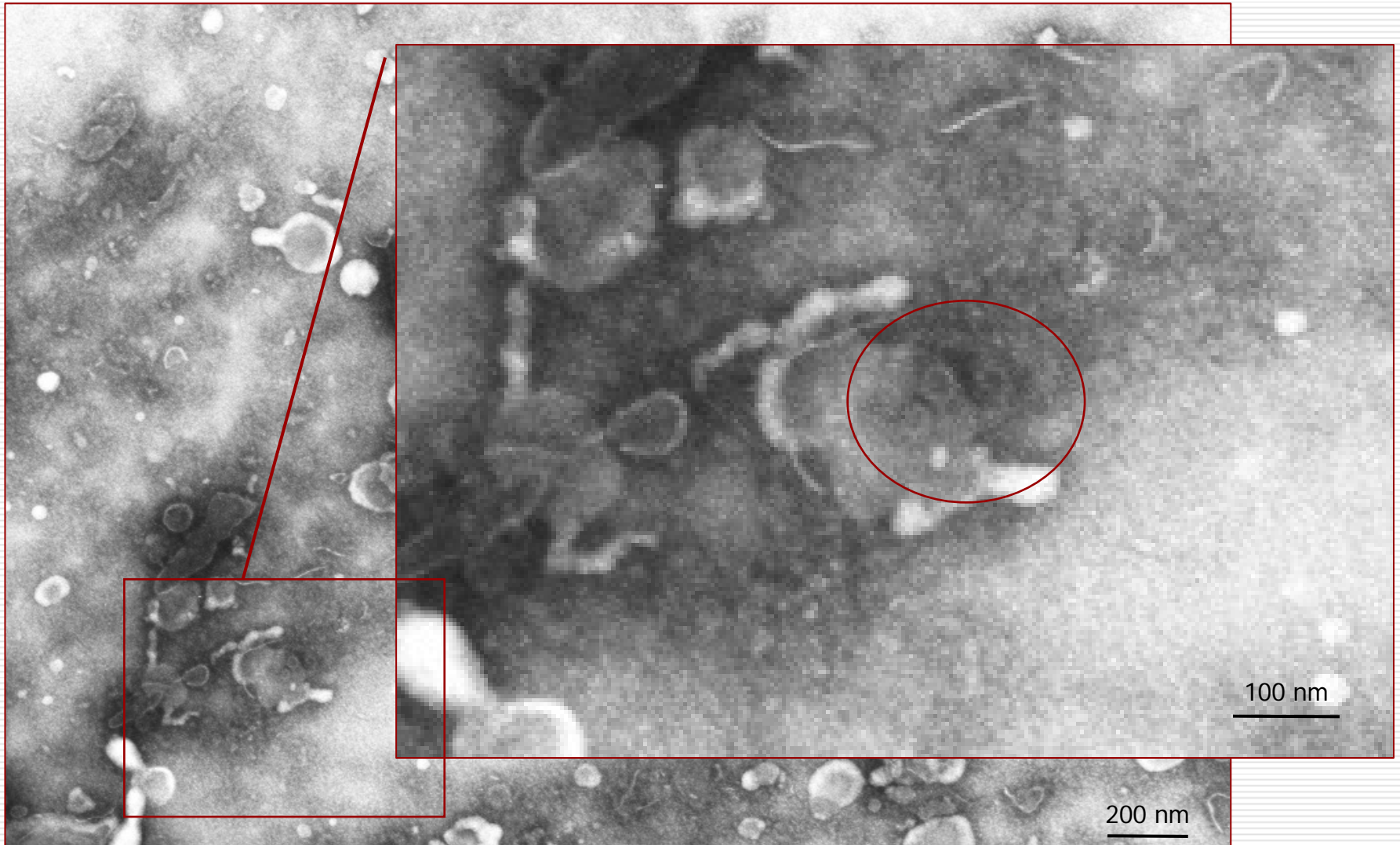
Emerged in Europe on '80 but the
aetiological agent was identified
(using IEM) on 1988.

Presently endemic only in Europe.

Morbidity 90-100%

Mortality 30-50%

nsEM: 1st “undetected” RHDV positive (Nov. 1986) from lung

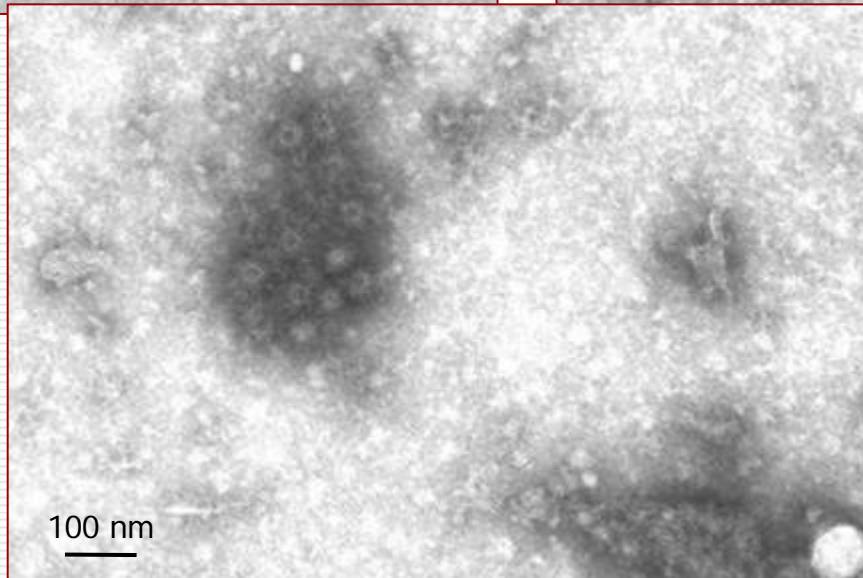
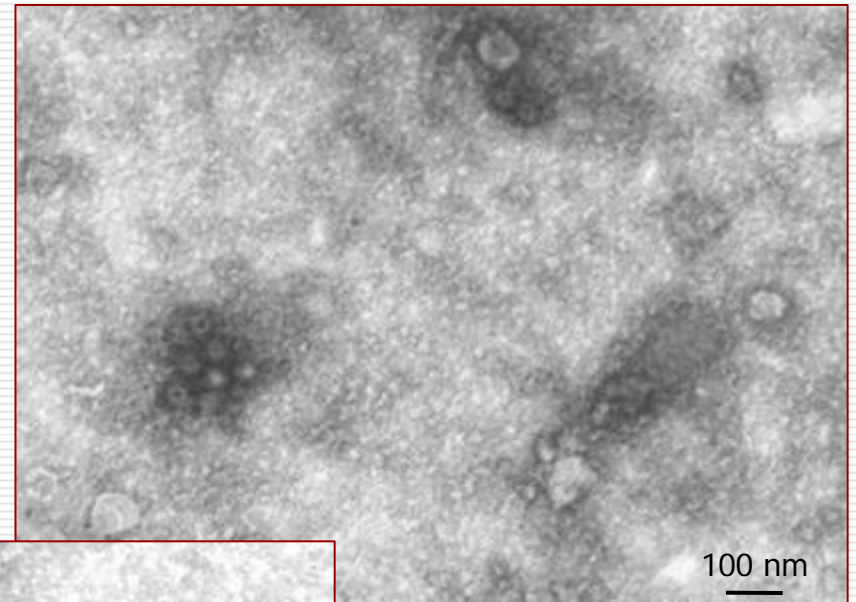
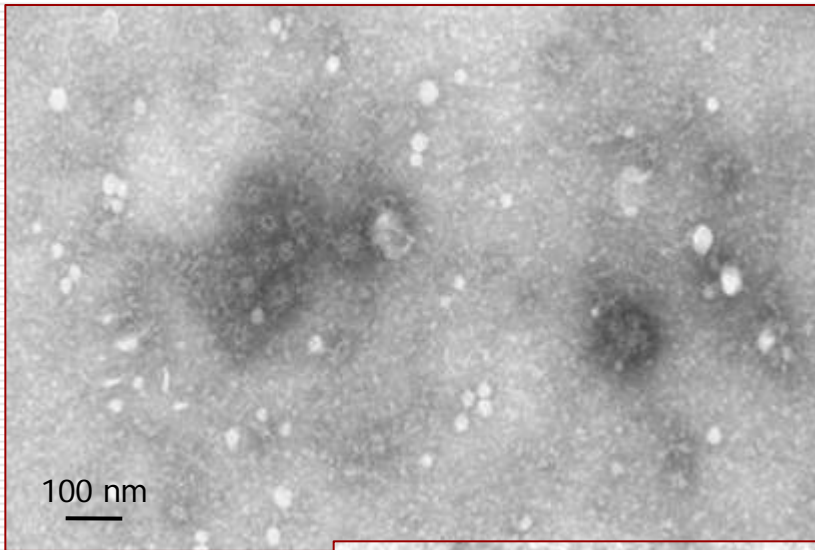


nsEM: 1st "detected" RHDV pos. (30-06-88) from liver

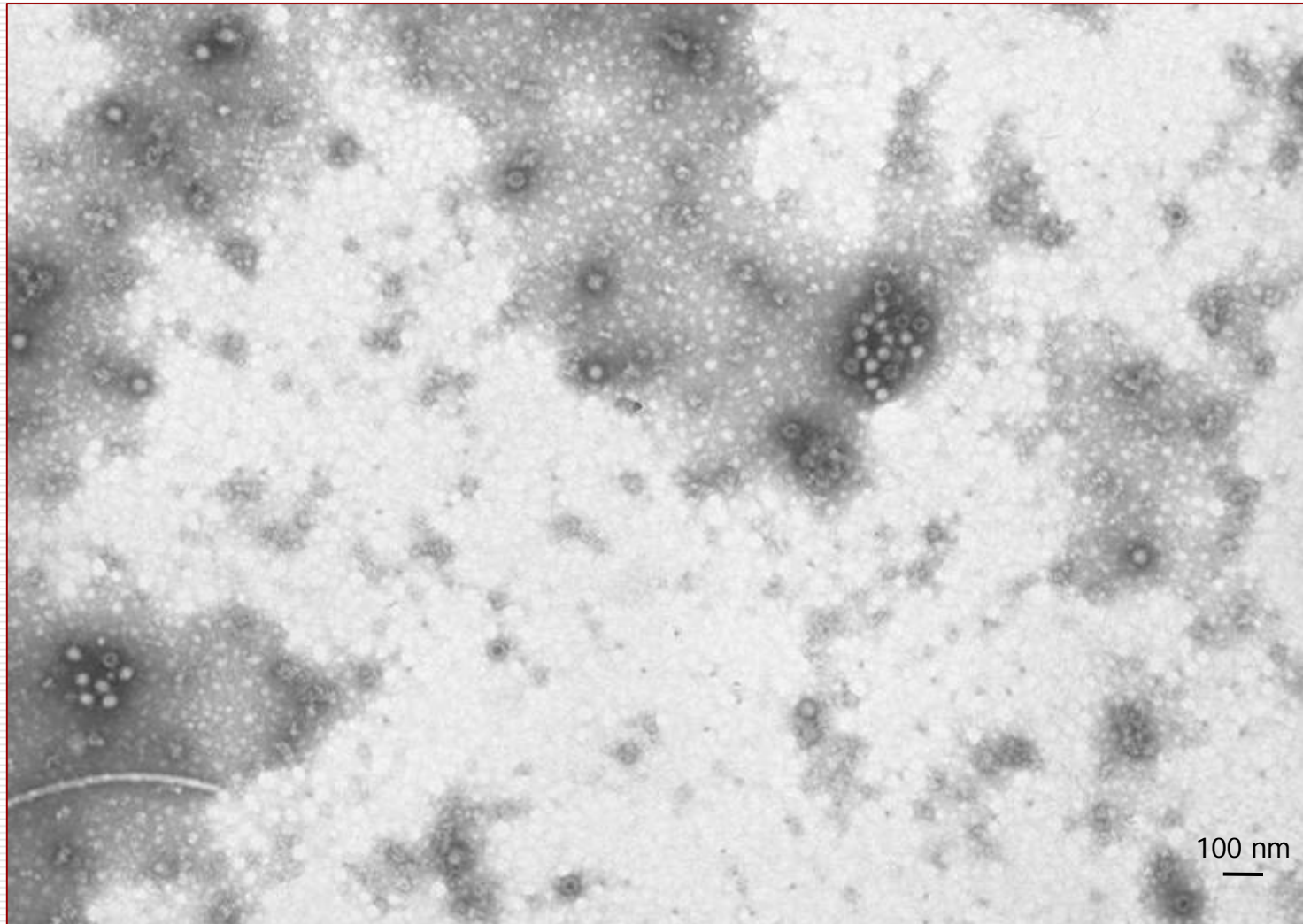


100 nm

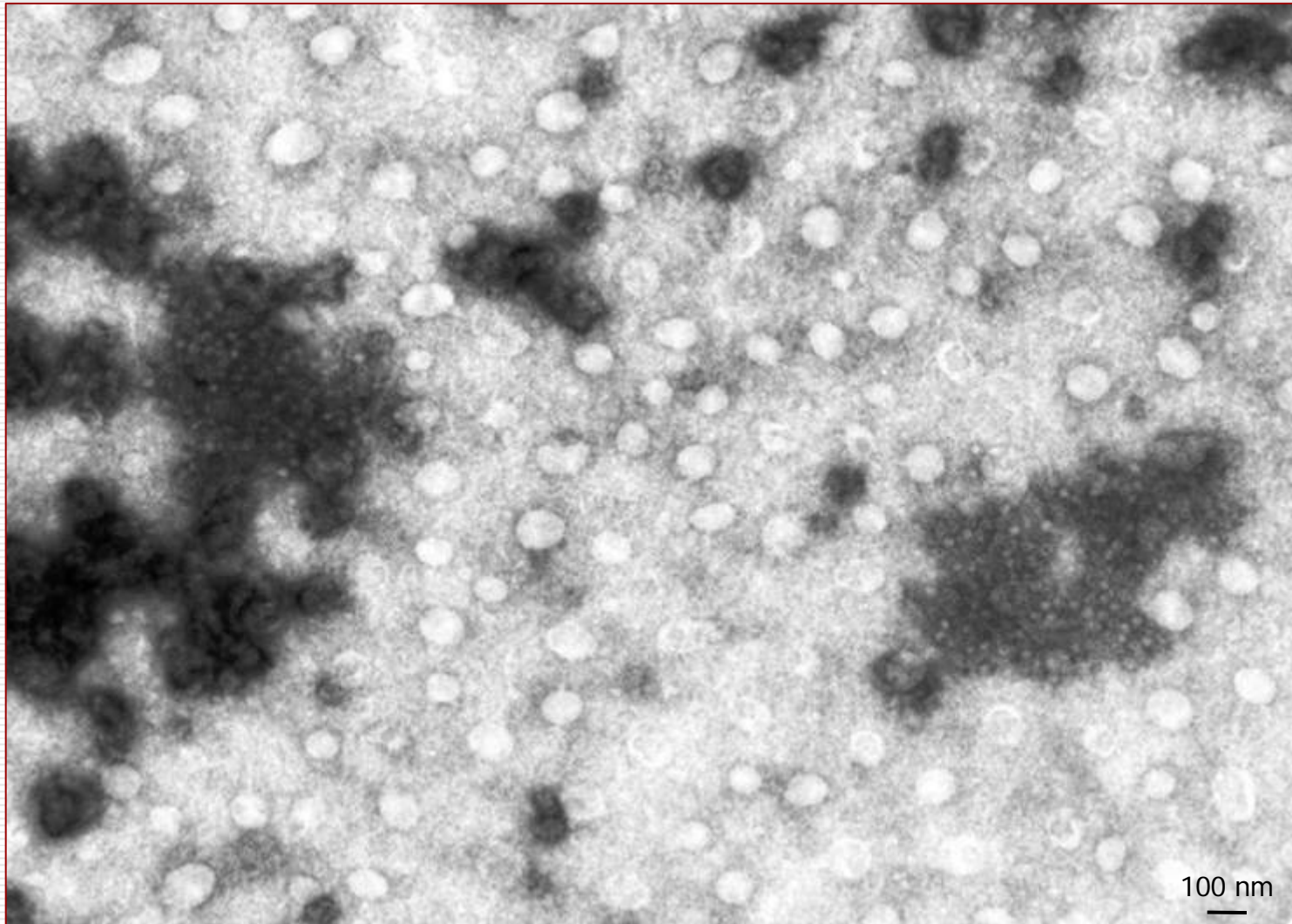
IEM: 1st “detected” RHDV pos. (30-06-88) from liver using a convalescent serum



IEM: 1st EBHSV positive 29-09-88 from liver
using an anti-RHDV convalescent serum

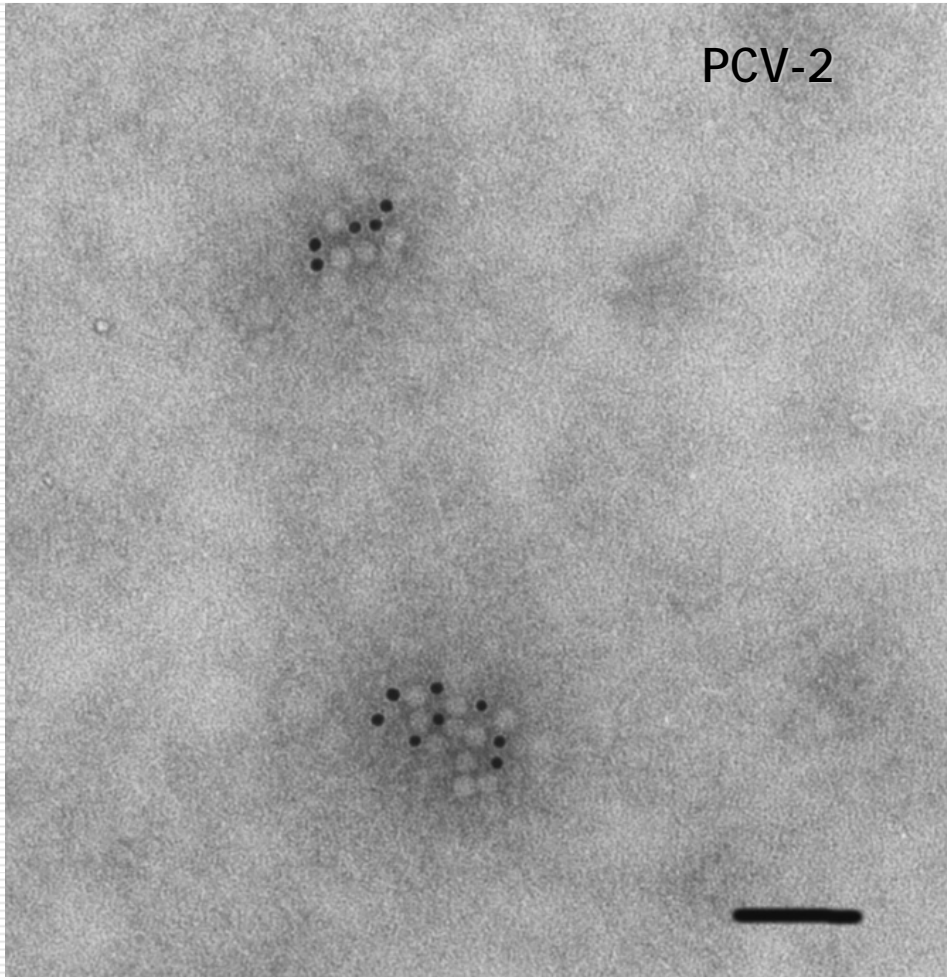


IEM: EBHSV pos. using an anti-EBHSV convalescent serum



100 nm

Colloidal gold IEM



By using and electrondense marker i.e. 5-30 nm colloidal gold particles

They are bound to antibodies by electrostatic link

Used as tracer of antibody binding

For the identification of particles:

- pleomorphic or not specific morphology
- present in low amount
- hidden by materials causing visual other interference

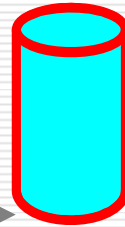
"Gold-labelling in suspension method"

(modified from Kjeldsberg, 1986 and Hopley, 1985)

1)

Tested samples
Freezing and
thawing cycles
Double
centrifugation
for clarification

50 μ l



50 μ l

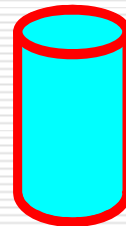
37°C for 1 hr
in humid atmosphere

Primary antiserum
(MAb or polyclonal)
diluted 1:40 in
Tris 0.02M (TB) +
0.1% BSA

2)

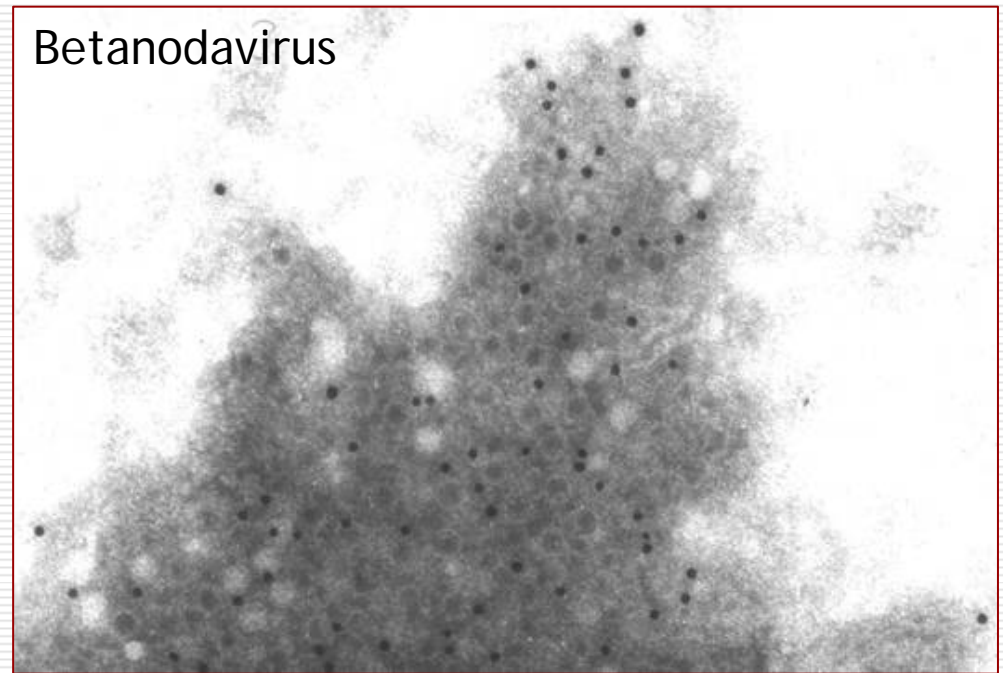
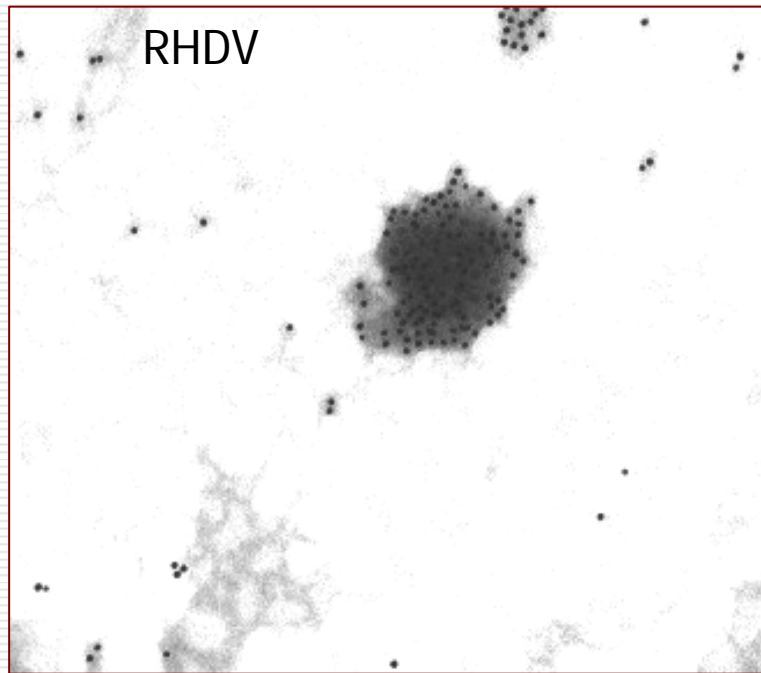
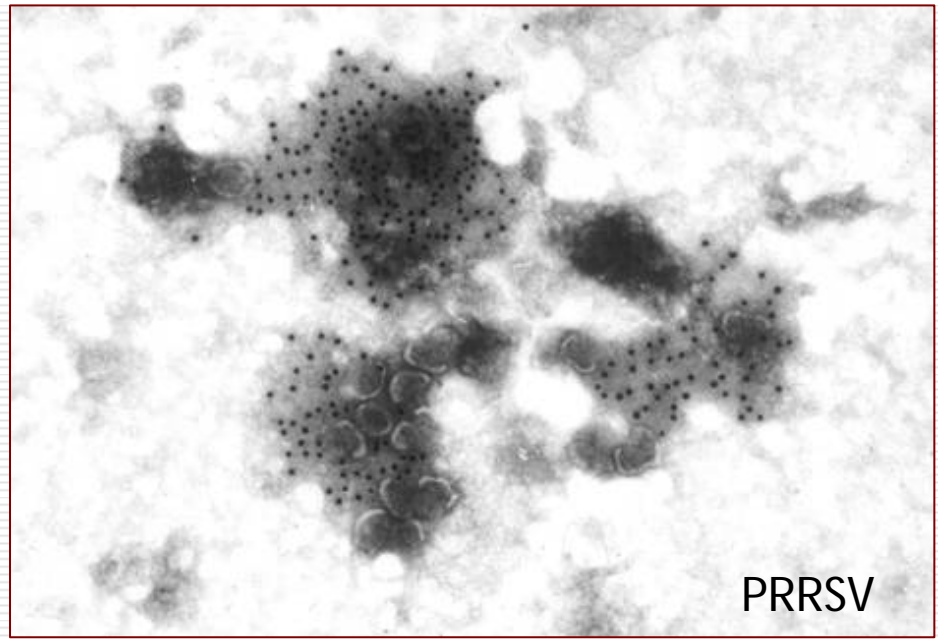
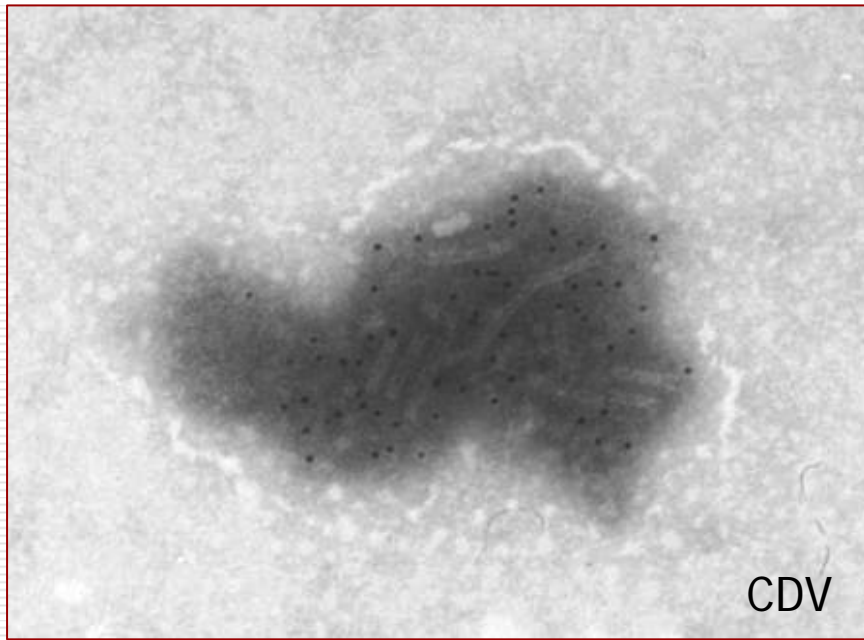
Gold-conjugated 10 nm
(Anti-mouse-gold
Protein A-gold)
dilution 1:5-1:40 in TB
+ 0.05% Tween 20
+ 0.1% BSA

50 μ l



37°C for 1 hr
in humid atmosphere

Steps
3), 4), 5)



Sampling indications

Samples should :

- always accompanied by full **anamnestic data**
- accurate and finalised to the **diagnostic suspect**
- taken just at the outcome of the first clinical signs (fever?)
- protected by **phisical-chemical damages** (high temperature, low or high pH values, exposition to disinfectants, sunlight, drying)
- maintained at **frozen T°C**
- conferred to the laboratory **in the shortest time**

False negative results

Morphological modifications due to:

- sampling mistakes
- incorrect conservation of samples
- animals sampled during chronic phase
- virions aggregated by Ab and destroyed
- subjectivity of examinations
- ability and experience of the operator

Lacking of anamnestic data:

- that hampers the application of IEM methods

Conclusions (1)

From our practical experience.....
.....nsEM methods and particularly IEM can be
usefully employed for the diagnosis of viruses
in animals

In fact, they permit to detect
not cultivable virions and multiple viral infection
in a very short time

In addition, thanks to
the undirected “**open view**” of conventional TEM
and its “**catch all**” property,
new, emerging or elusive viruses
may be identified

Conclusions (2)

By looking at our publications.....

.....the results obtained by using nsEM as diagnostic methods may be the base for further studies on:

- antigenic and molecular characterization of viruses
- pathogenesis of infections
- epidemiology of diseases

Thanks for your attention



Grazie per il loro contributo a tutti i collaboratori

- ***Microscopia Elettronica – IZSLER***
- ***Microscopia Elettronica e Virologia Speciale - IZSLT***