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Detection of Equine Viral Diseases –
Considerations for sampling

Training on Laboratory Techniques for Equine Diseases
29th – 30th Nov 2021

Falko Steinbach

Mammalian Virology Group, APHA, Weybridge, UK

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APHA

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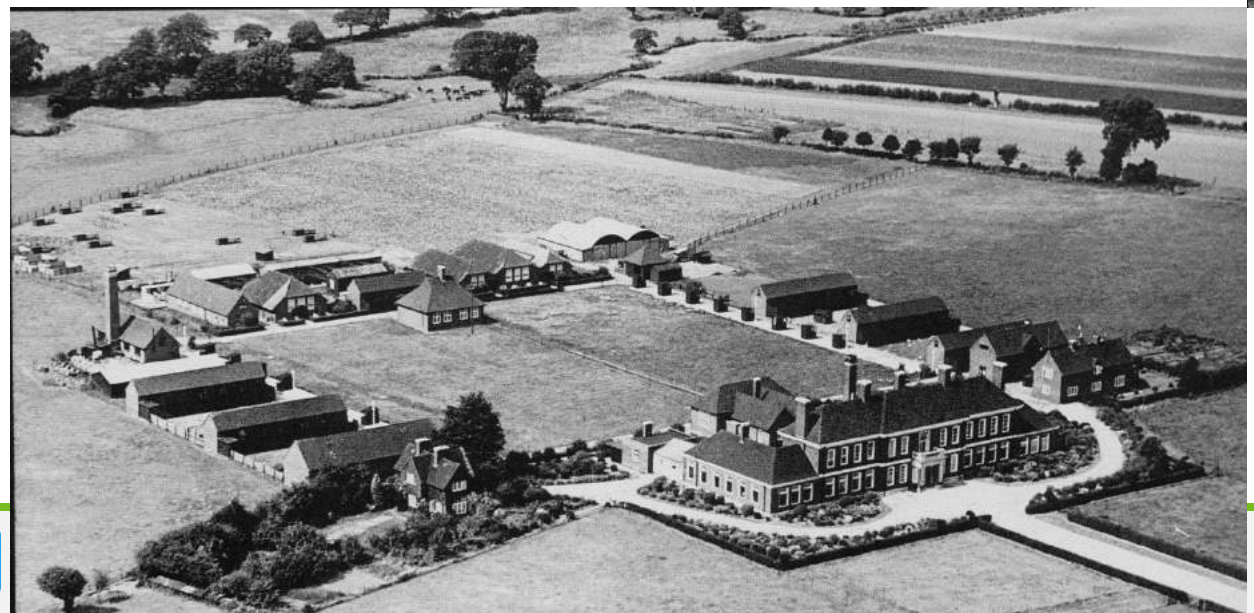
APHA - A brief history

1917 - The Addlestone Institute opens
1922 - Veterinary Investigation Service (VIS) established
1990 - Central Veterinary Laboratory (CVL) launched
1995 – Veterinary laboratories Agency (VLA) launched
now including “regional laboratories”

2011 - **AHVLA launched following the merger of VLA and Animal Health**

2014 - APHA following the merger of AHVLA and FERA



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- Specialist facilities for:
  - **Research**
  - Animal studies
  - Large scale testing
  - Reagent and product manufacture

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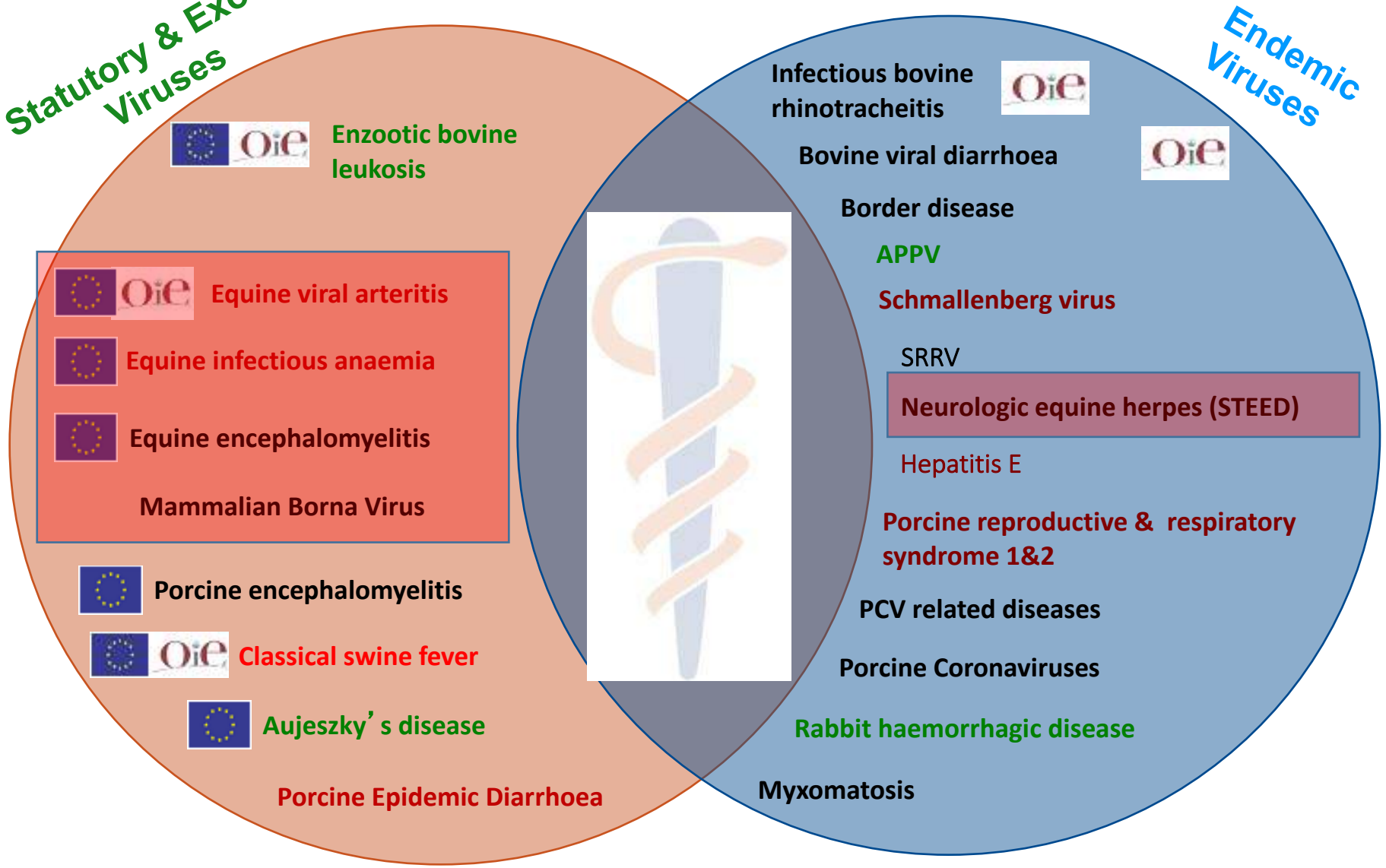
Mammalian Virology APHA



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Horses have diseases of their own but also carry zoonotic infection

- **viral diseases**
  - West Nile Virus
  - Rabies
  - Vesicular Stomatitis Virus
  - Equine Encephalitidis Viruses (VEEV, EEE, WEE)
  - Borna Disease Virus
  - Equine Viral Arteritis (EVA)
  - Equine Infectious Anemia Virus
  - Equine Influenza
  - Equine Rhinopneumonitis (EHV-1/-4)
  - African Horse Sickness virus
  - Horsepox
- **bacterial diseases**
  - Burkholderia mallei (Glanders)
  - Bacillus antracis
  - Taylorella equigenitalis (CEM)
- **parasitic diseases**
  - Trypanosoma equiperdum (Dourine)
  - Trypanosoma evansi (Surra); [DD T. brucei]
  - Theileria equi (Piroplasmosis)
  - Babesia caballi (Piroplasmosis)

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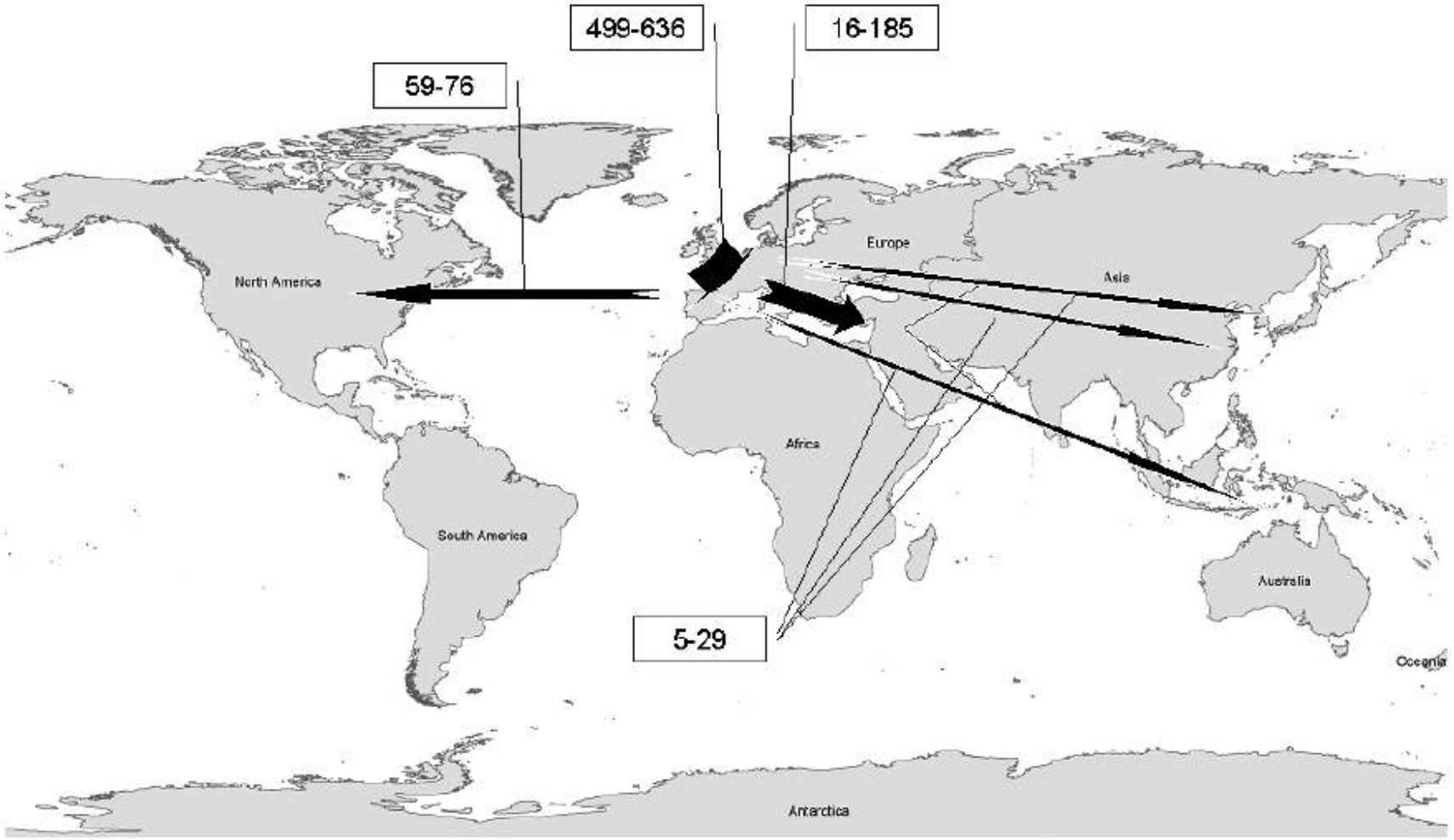

Fig 2: Worldwide movements of racehorses between 2001 and 2006. The figure shows the range of the number of movements during that time period, the thickness of the arrows indicate the number of movements in 2006.

Herholz et al., EVJ, 2008

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No other living animal travels as much and far as horses

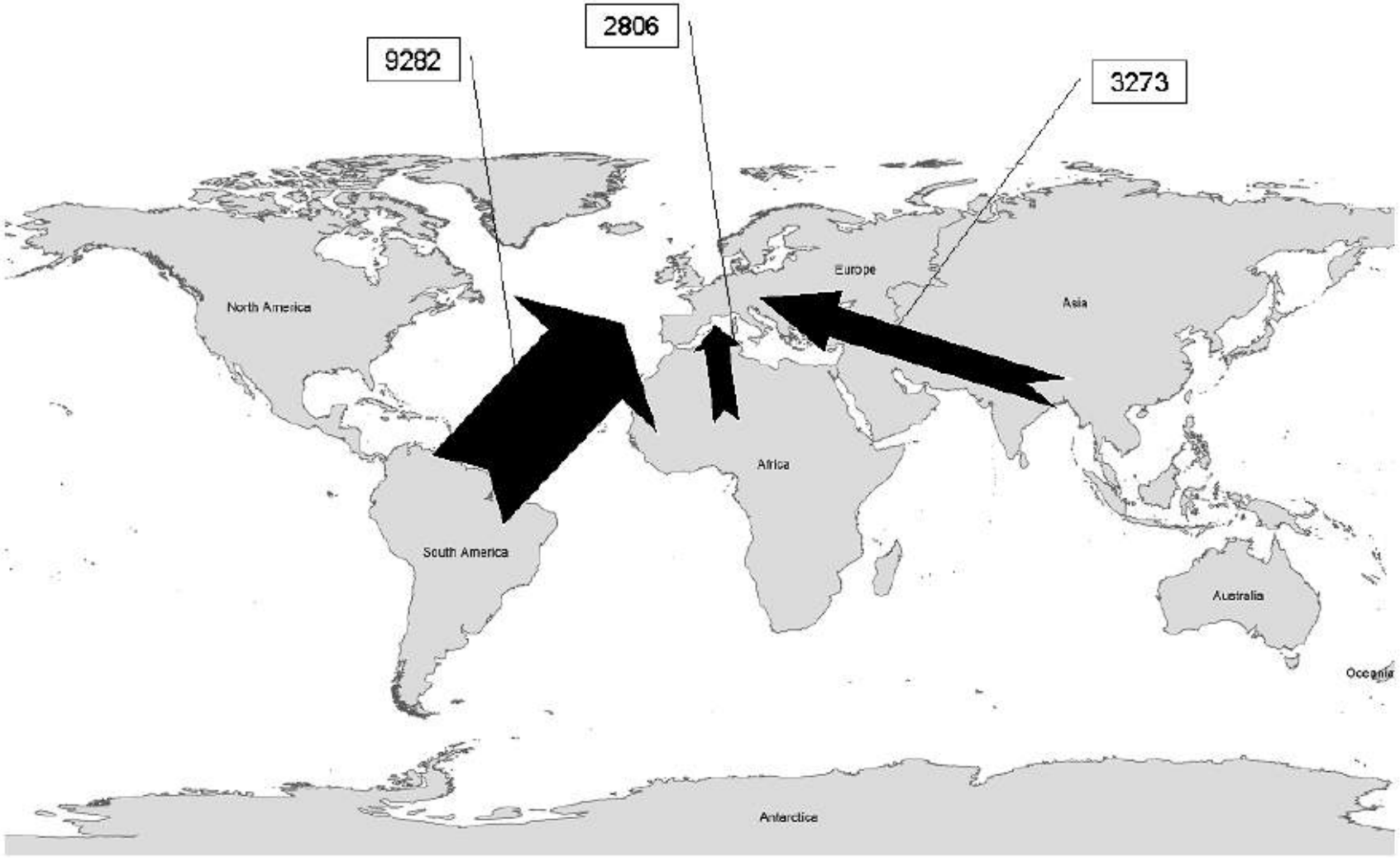

Fig 3: Movement of horses from Africa, Asia and South America to Europe. The figures and the thickness of the arrows represent the sum of movements during that time period (TRACES data from 2004 to summer 2007, Table 3).

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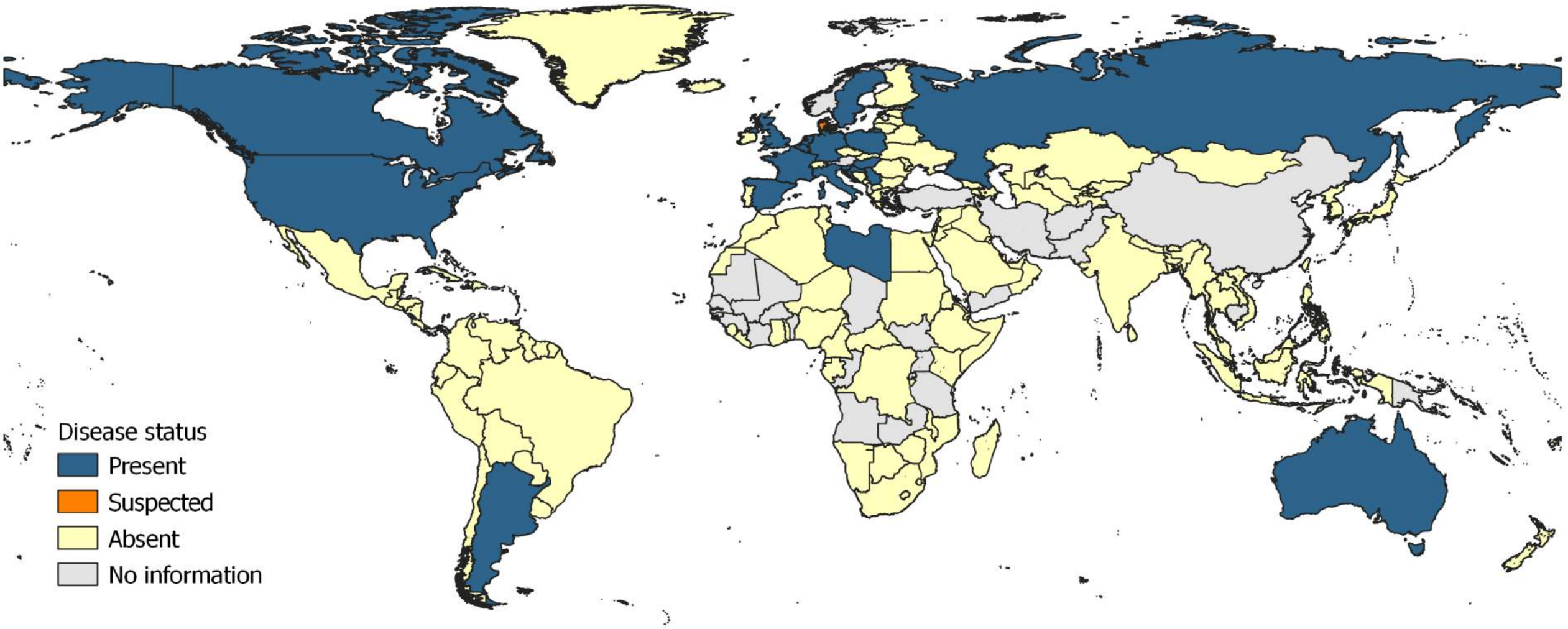
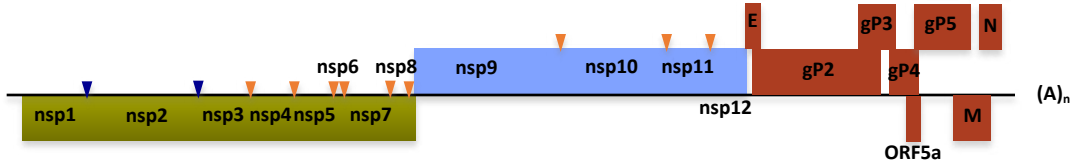
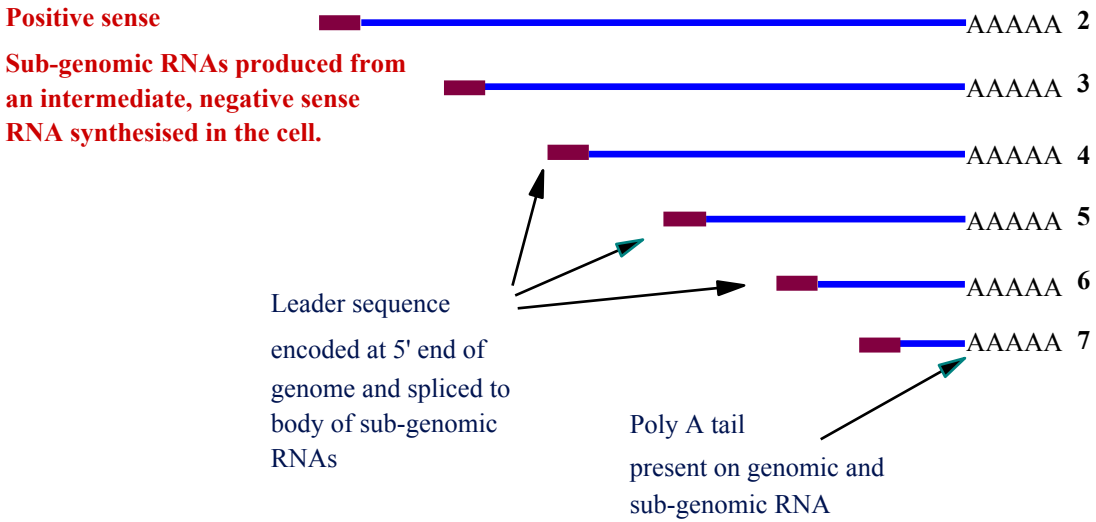
EAV 2016-2020

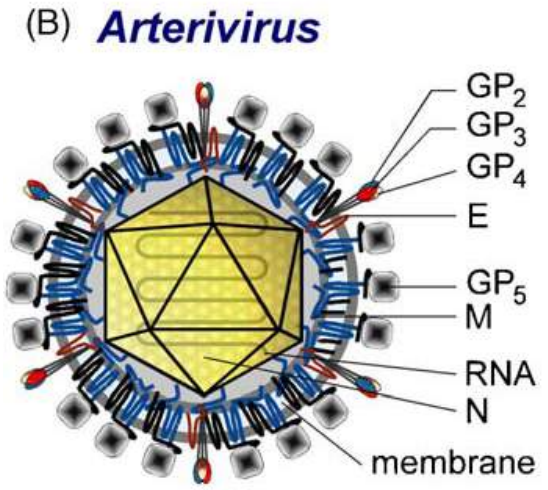


Equine Arteritis Virus (Nidovirales)

Genomic RNA positive sense, approx. 12.7kB







Related to PRRSV

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Clinical presentation and regulation in the UK

- **Incubation ≈ 7 days - no signs**
- **Signs vary considerably - greater in old and young**
  - **Fever**
  - **Oedema**
    - **legs, brisket, scrotal/mammary, jaw, periorbital**
    - **skin plaques with urticaria**
    - **severe bilateral conjunctivitis & lacrimation**
  - **Upper respiratory tract infection**
  - **Depression/anorexia**
  - **Skin rash, mostly on neck**
  - **Abortion**
  - **Death in young, old and immuno-compromised animals**
- **Suspicion of clinical case or carrier animals (stallions) is notifiable to the authorities**

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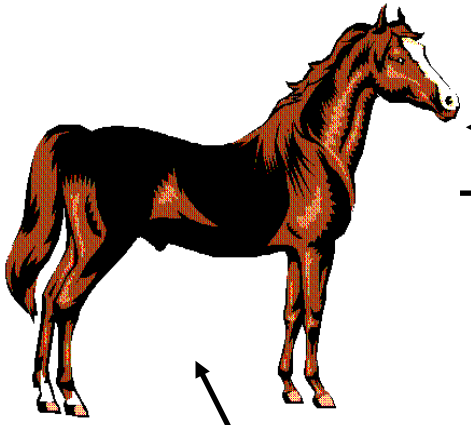
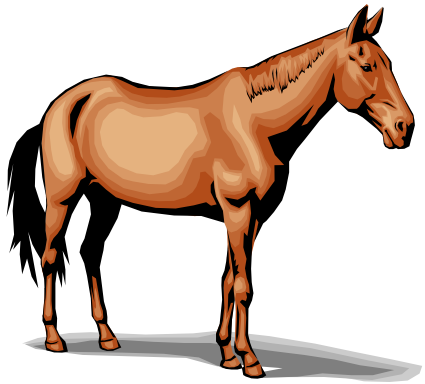
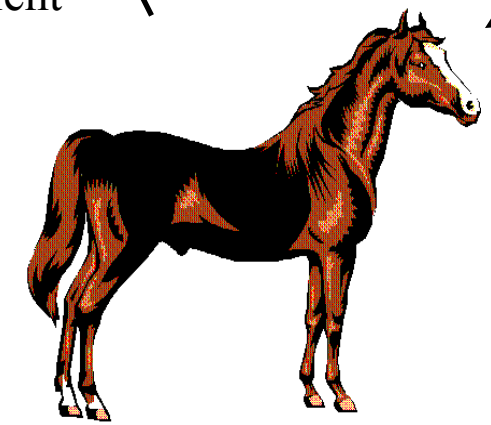


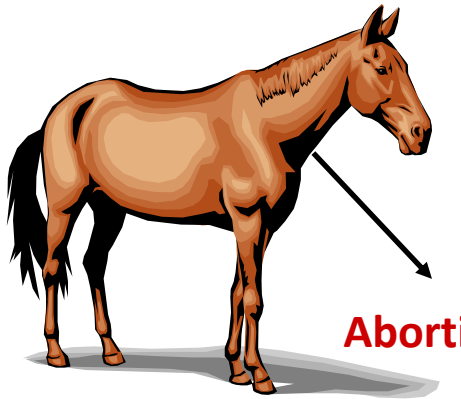
Venereal route
artificial or natural



Mare sheds large amount of virus - respiratory, urine, colostrum



Respiratory route



Abortion

Persistence

May be transient or lifelong

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B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of equine viral arteritis and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection	Efficiency of eradication policies	Confirmation of clinical cases	Prevalence of infection - surveillance	Immune status in individual animals or populations post-vaccination
Virus isolation	–	+++	–	+++	–	–
Agar gel immunodiffusion	–	–	–	–	–	–
Complement fixation	–	–	–	+++	–	–
Enzyme-linked immunosorbent assay	+	++	+	++	+++	+
Polymerase chain reaction	–	+++	–	+++	–	–
Virus neutralisation	+	+++	+	+++	+++	+++

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

Terrestrial Manual

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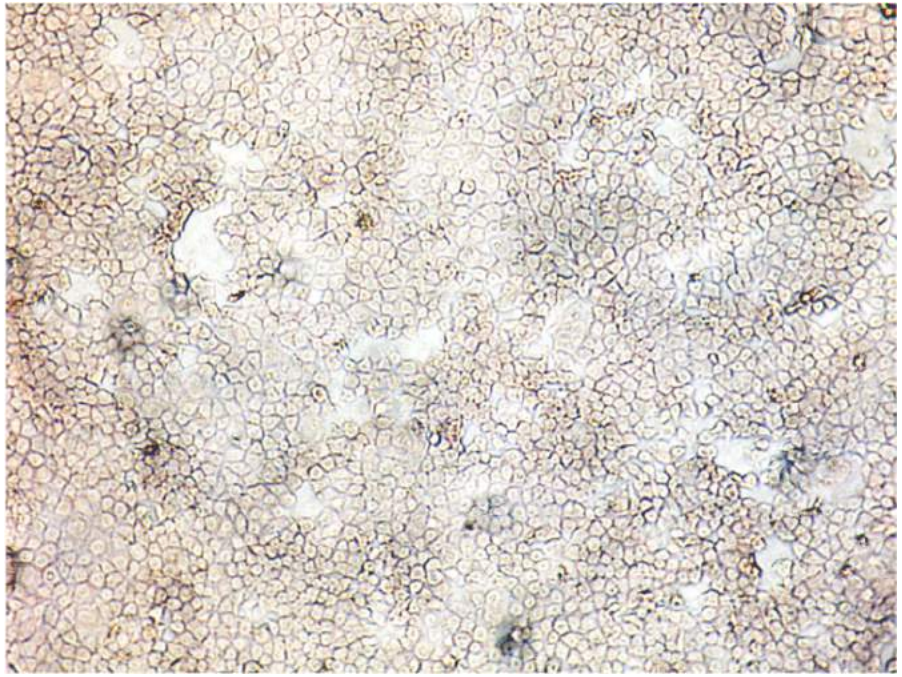
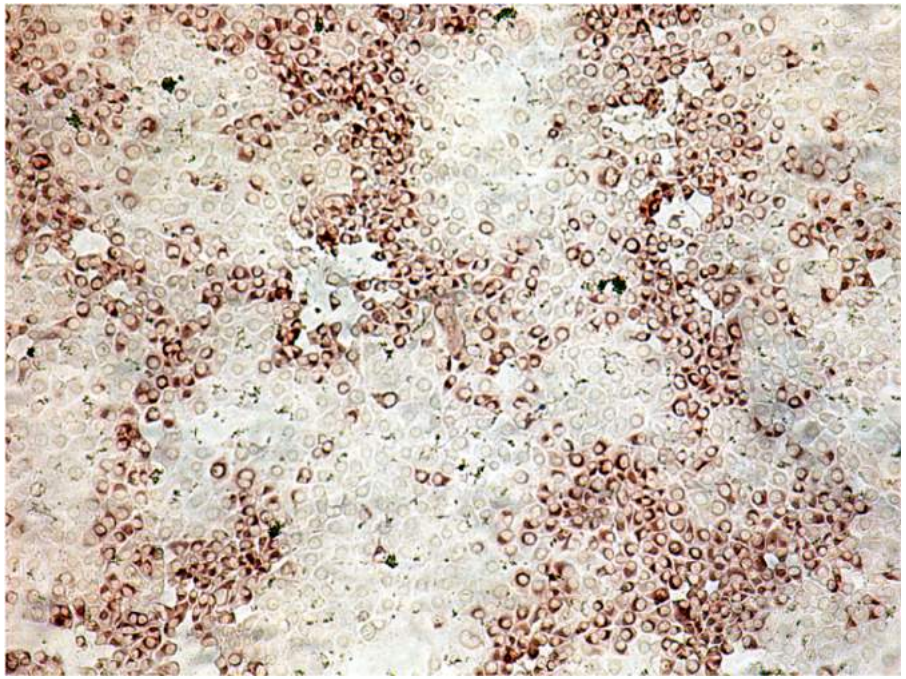
Methods applied for EVA investigations





Virus isolation (RK-13)

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UK 2011 (62)

- **Imported from the continent ~ 3y before**
- **Tested prior to breeding**
  - **SNT 1:4096**
- **VI negative**
- **PCR/qPCR positive**
  - **ORF 1; -5; -7**

- semen analysis for nAbs
  - Including inactivation (virus) & negative control semen to discard for inhibitory factors

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nAbs in semen – not a unique phenomenon

Horse	Serum VN-titre	Semen VN-titre
1715	>2048	64
No2 2010	3072	32
62/167	4096	256

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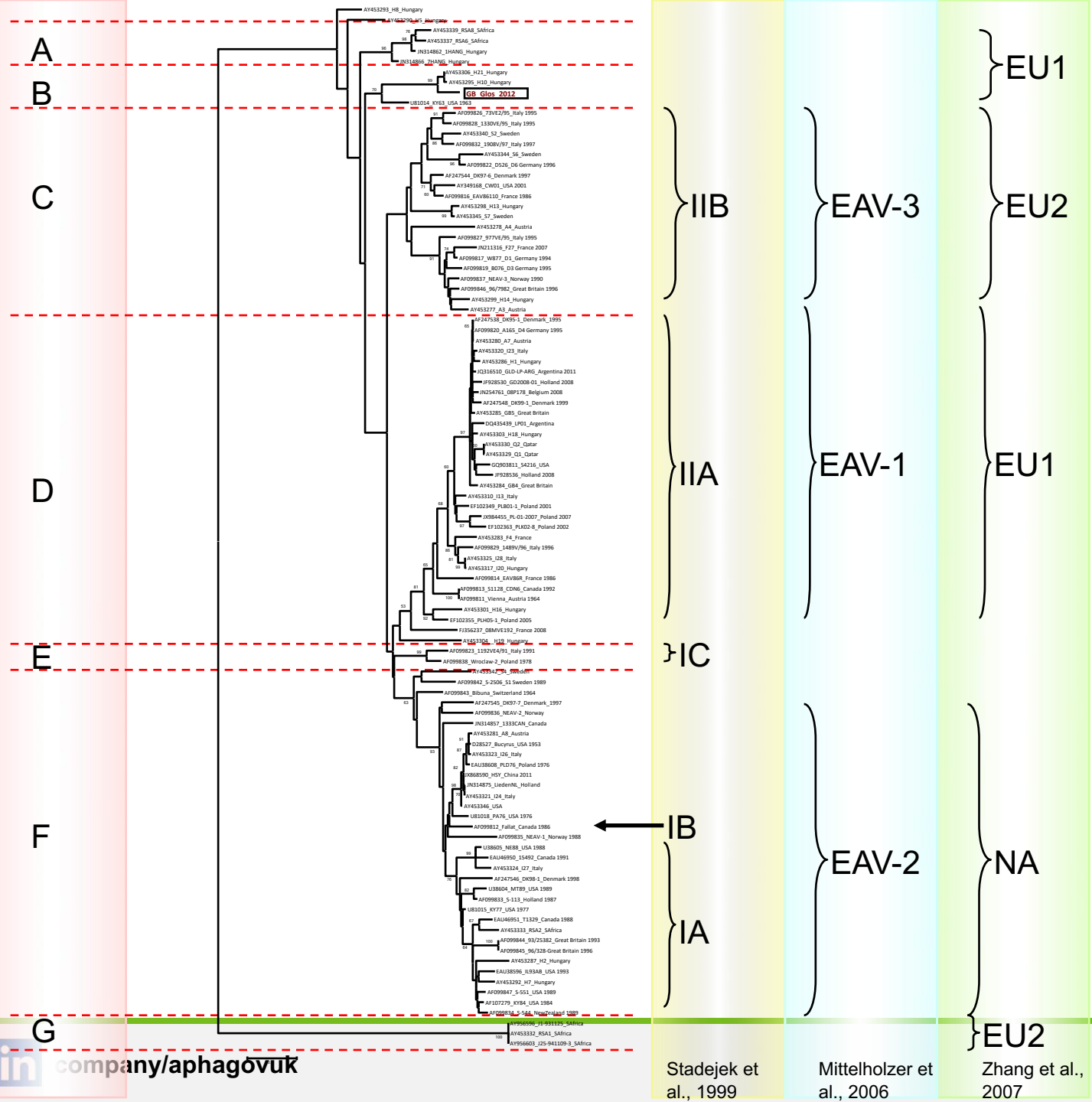
ORF5



Steinbach F, et al. (2015)
Virus research, **202**: 144-150
doi.org/10.1016/j.virusres.2014.12.009

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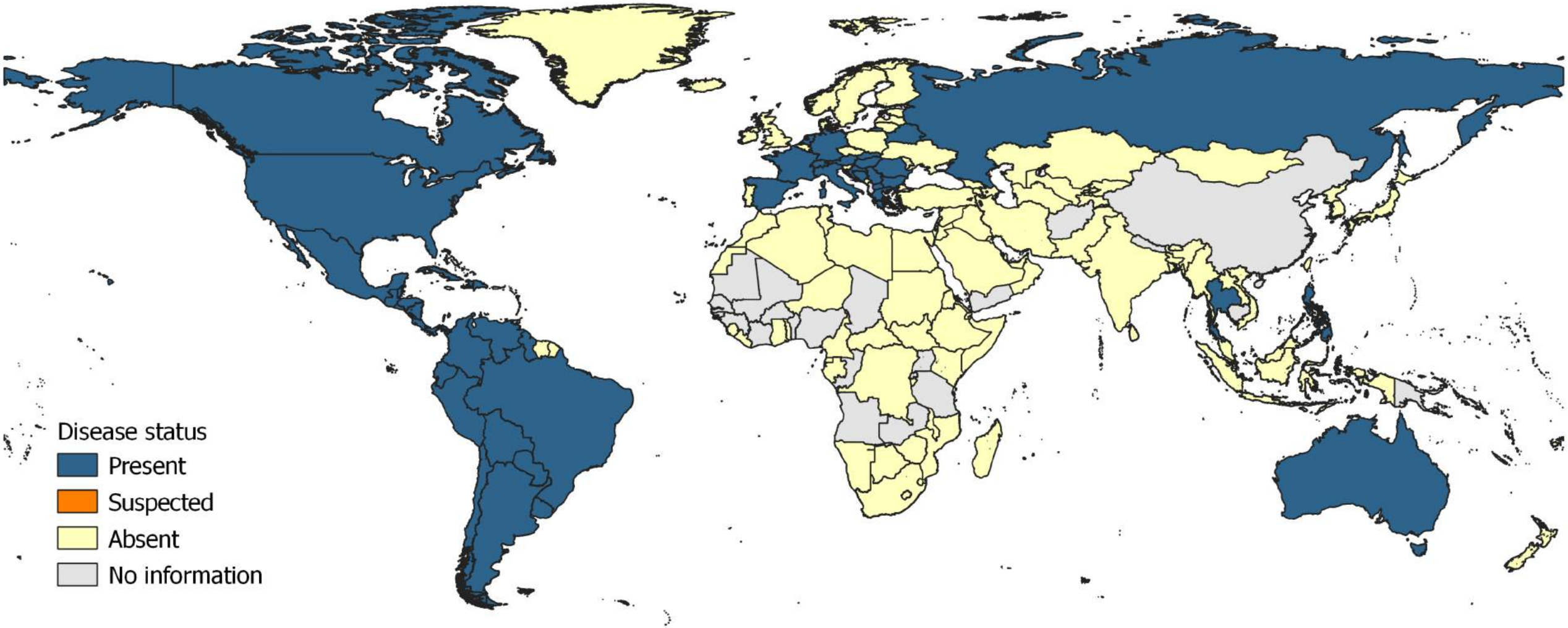
EIA 2016-2020



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The disease:

- **Infectious and potentially fatal (horses only) viral disease for equids (horses, donkeys, mules).**
- Increased prevalence in warm humid environments.
- Torrance (Canada) crafted the term "**swamp fever**" in 1903.
- **Acute**, **Chronic**, and **Inapparent** forms

Bucky is a 35-year-old (2006) Equine Infectious Anemia (EIA) positive gelding that has lived at Malden Brook Farm in West Boylston, Massachusetts since 1984.

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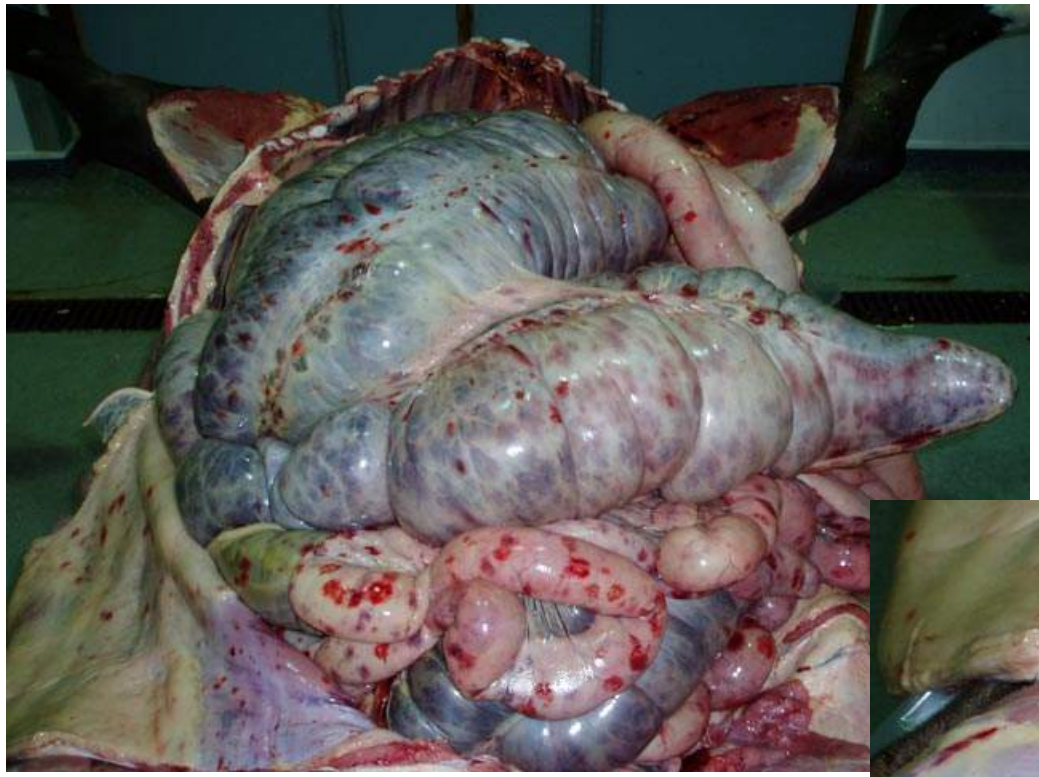
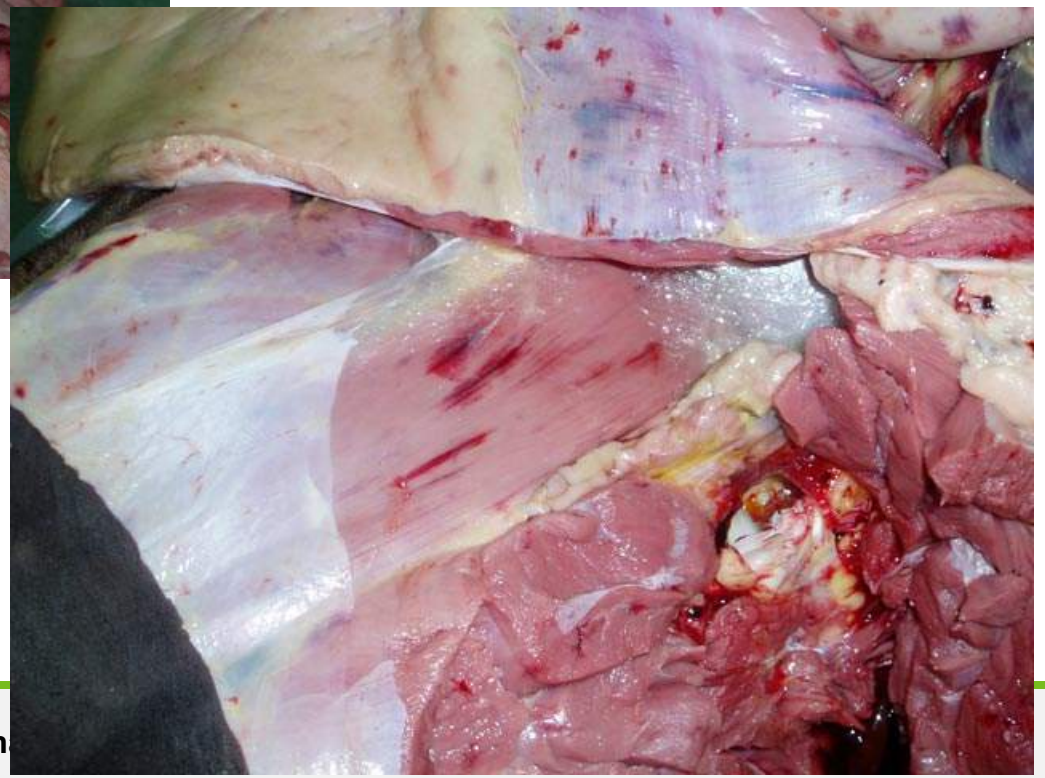
Post mortem findings



Case Germany, 2006



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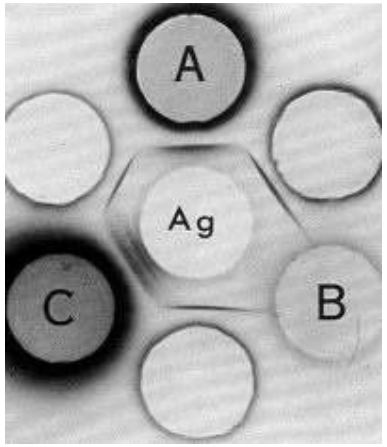
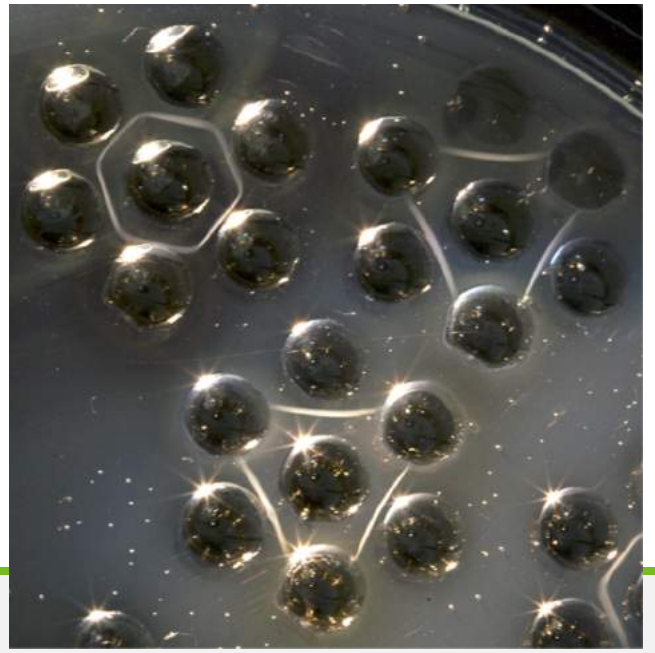
Laboratory tests

- **Coggins test -**
  old, safe, late, time consuming
- **ELISA – some validated**
- **Western blot – a necessary addition**
- **PCR - an interesting problem**





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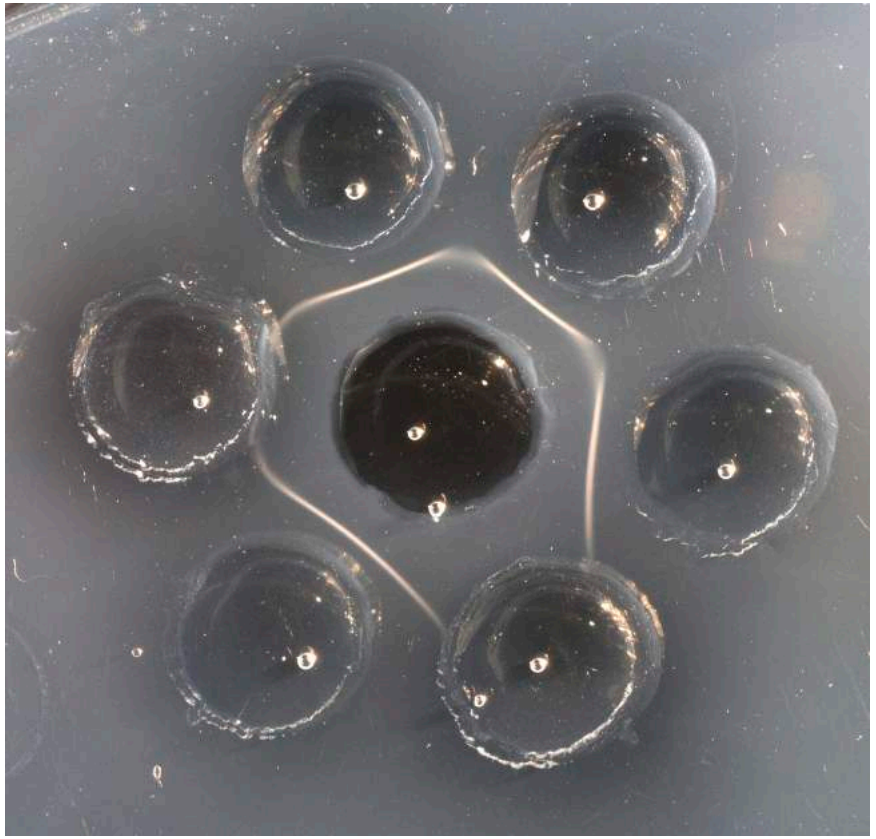
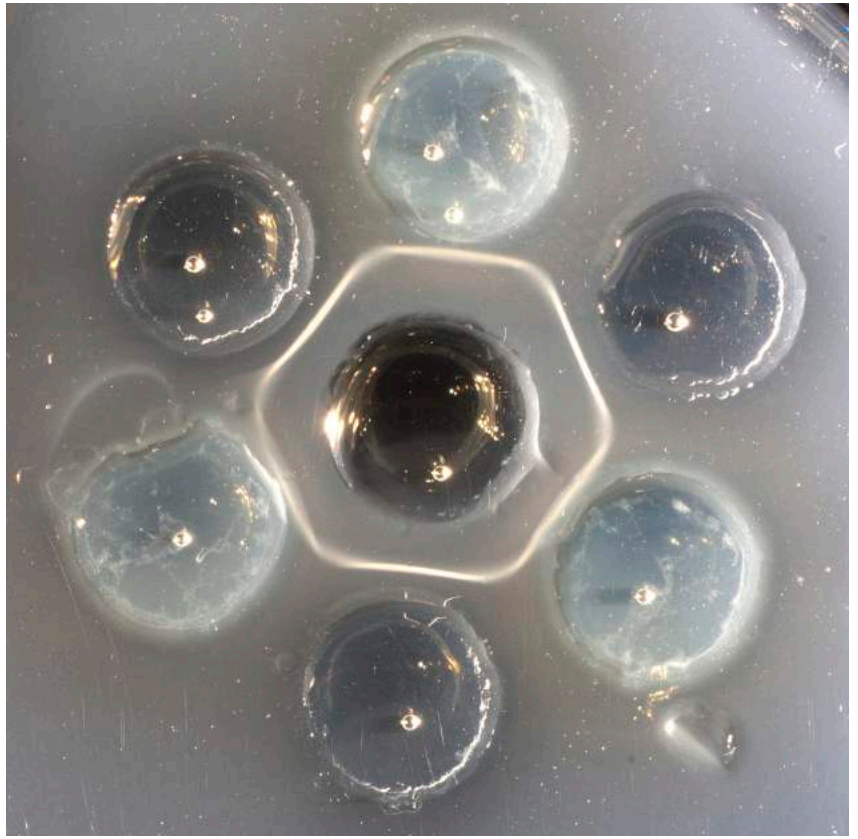
Coggins





control **case**

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ELISA:

- Several ELISAs are currently approved by various national bodies in the USA and Europe and are available internationally. For example:
  - A competitive ELISA and a non-competitive anti-p26 Abs.
  - Another non-competitive ELISA uses both p26 core and gp45 (viral transmembrane protein) antigens.
- A positive test result by ELISA should be confirmed using the AGID test because individual false-positive results have been noted with the ELISA.
- If there is discordance between the two techniques, the results can also be confirmed by the immunoblot technique.

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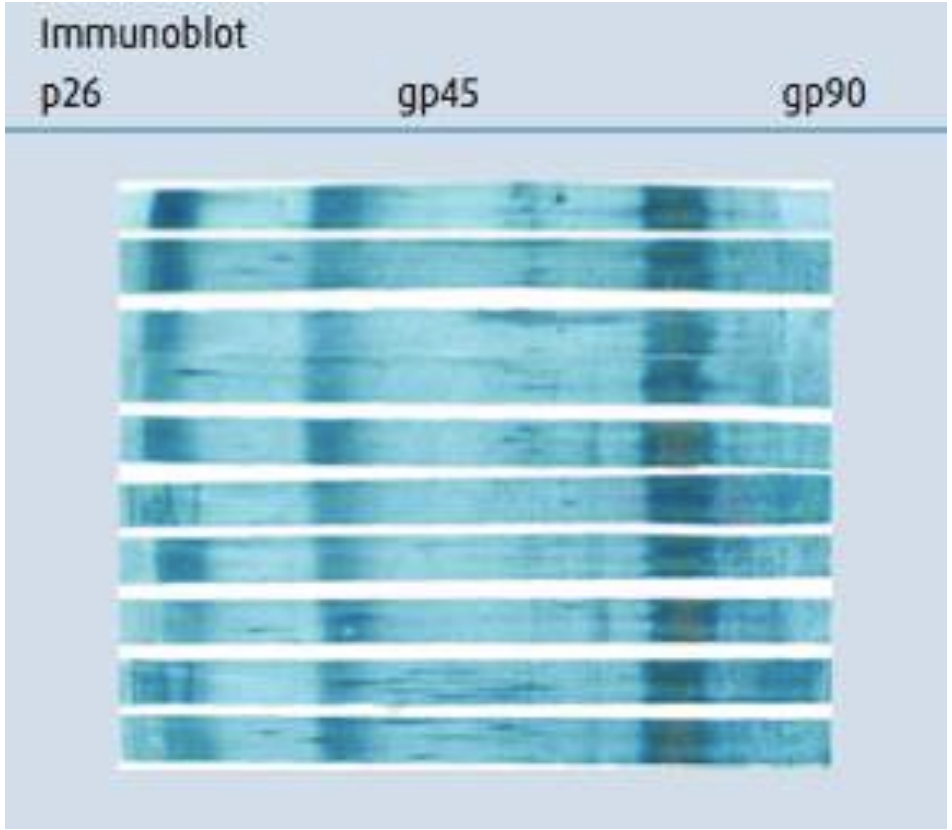
Immunoblot



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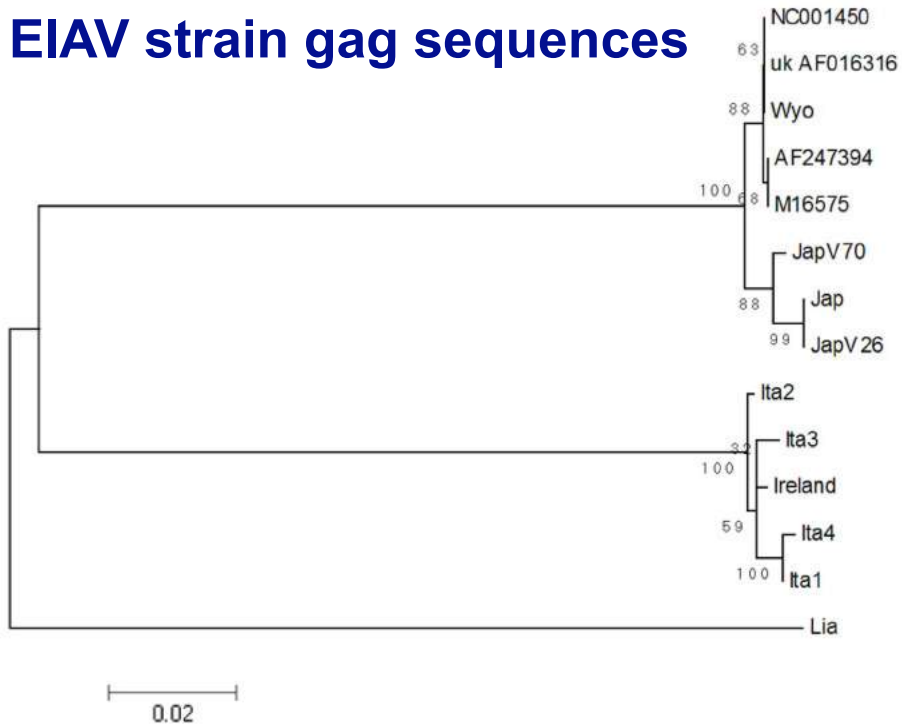
EIAV genetic diversity

EIAV strain gag sequences



Long-term nonprogressors (LTNPs) HIV

Viraemic controllers have low but readily measurable virus loads

Elite controllers suppress to extremely low levels

The detection level has to be very low ……

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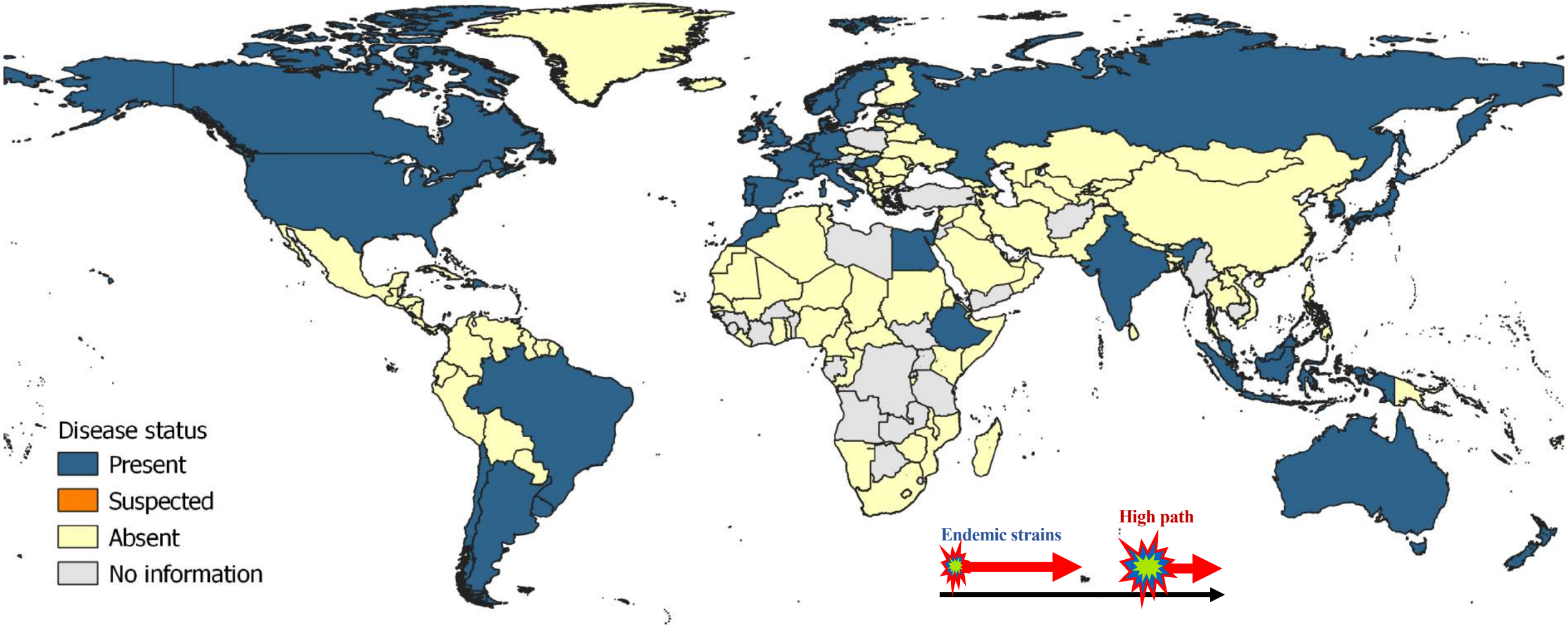
EHV-1 2016-2020



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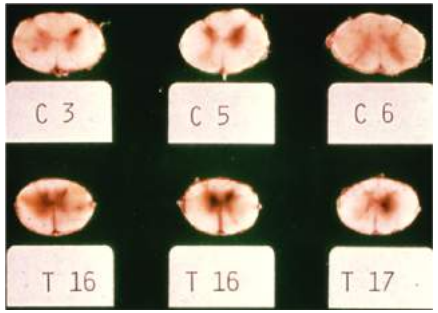
Neurotropic EHV-1 infection

Equine herpes virus myeloencephalopathy



The severity of clinical signs is variable

- hindlimb incoordination to quadriplegia - paralysis

- urinary & fecal retention
- loss of sensation at the perineum and tail

Chowdhury et al., 1986
Equine herpesvirus type 1 (EHV-1) induced abortions and paralysis in a Lipizzaner stud
An event that threatened to kill the Lipizzaner breeding in Austria (1983)
30 cases of abortion and perinatal deaths in a Lipizzaner stud in Austria
10 mares die after having shown central nervous system disturbances, ataxias and paralysis

2007:
EQUINE HERPES VIRUS (EHV) KILLS 40 TOP POLO PONIES IN NIGERIA!

2021
EHV-1 outbreak at show jumping event in Spain kills several animals and causes disruption to the FEI calendar

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A series of publications has suggested that a single point mutation in the DNA polymerase (ORF30) of equid herpesvirus 1 (EHV-1) determines neurovirulence

Nugent et al., JOURNAL OF VIROLOGY, Apr. 2006, p. 4047–4060

Analysis of Equid Herpesvirus 1 Strain Variation Reveals a Point Mutation of the DNA Polymerase Strongly Associated with Neuropathogenic versus Nonneuropathogenic Disease Outbreaks

Hypothesis: It has been suggested that **distinct strains of EHV-1 that differ in pathogenic capacity** circulate in the field. In order to investigate this hypothesis, it was necessary to identify genetic markers that allow subgroups of related strains to be identified.

Results The results indicate [A] the occurrence of several major genetic subgroups of EHV-1 ..... consistent with the proposal that distinct strains of EHV-1 circulate in the field.

. [B] that certain strain groups are geographically restricted, being recovered predominantly from ..... either North America or Europe. .....

[C] variation of a single amino acid of the DNA polymerase is strongly associated with neurological versus nonneurological disease outbreaks.

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Further reports

USDA-APHIS (2007) **Equine herpes virus myeloencephalopathy: A potentially emerging disease. http://www.aphis.usda.gov/vs/ceah/cei/taf/emergingdiseasenotice_files/ehv.pdf. Accessed 21 Jan 2008**

Goodman, L. B., A. Loregian, G. A. Perkins, J. Nugent, E. L. Buckles, B. Mercorelli, J. H. Kydd, G. Palu, K. C. Smith, N. Osterrieder, and N. Davis-Poynter. 2007. A point mutation in a herpesvirus polymerase determines neuropathogenicity. PLoS Pathog **3:**e160

author's summary:

Our report provides evidence for a direct causal link between the genotype of EHV-1 strains and their neurovirulence, and thereby gives a long-awaited explanation for the conundrum of the different clinical outcomes following EHV-1 infection. We proved that alteration of one amino acid in the key viral enzyme, DNA polymerase, which is conserved in all herpesviruses, renders the virus unable to cause neurologic disease.

PROBLEM: the clinical signs observed were very mild…….

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Goodman et al.

Table 1. Neurologic Grades, CSF Cytology, and qPCR

N=8 per group !

Animal ID	Group	Days Postinfection	Neurologic Grade	CSF Color	Nucleated Cells/ µl CSF	Differential Cell Count	CSF Total Protein (mg/dl)	CSF Viral Genome Copies/ml
Reference[a]	N/A	N/A	N/A	Clear	<6	No abnormalities	<100	0
4	D	7	2	Clear	5	75% lymphocytes; 25% macrophages	45	174
4	D	16	2	Yellow	56 LS; 24 AO	AO: 86% lymphocytes; 14% macrophages	215 LS; 139 AO	2,821 LS; 9,450 AO
9	D	16	1	Clear	2	53% macrophages; 47% lymphocytes	67	<5
42	D	9	0	Clear	0	No abnormalities	20	<5
228	D	7	0	Clear	9	80% small lymphocytes; 20% macrophages; few red blood cells	50	168
120	N	15	0	Clear	1	No abnormalities	67	<5
263	N	15	0	Clear	9	70% lymphocytes; 29% macrophages; 1% neutrophils	73	100
263	N	19	0	Light yellow	13	LS: 52% lymphocytes; 40% macrophages; 8% neutrophils	233	120 LS; <5 AO

Xanthochromia, elevated nucleated cell count, and elevated protein levels are typical (but not specific) for EHV-1 myeloencephalopathy [38]. Samples were collected from the lumbosacral (LS) space in the standing horse, and additional postmortem samples were collected from the atlantooccipital (AO) space. The four veterinarian reviewers' consensus hind limb neurologic grades are reported according to the scale from Mayhew et al. [39], with a grade 2 representing moderate pelvic limb signs, grade 1 representing mild signs, and grade 0 within normal limits. Grades for horses not listed in this table were zero.

*

*** Ab4 does not nec induce neurologic symptoms either**
Chong & Duffus 1992 1/4 animals mild neurologic symptoms (SPF)
Lunn et al., 1991 etc SPF foals w/o neurologic symptoms

Sutton G, Normand C, Carnet F, Couroucé A, Garvey M, Castagnet S, et al. Equine Herpesvirus 1 Variant and New Marker for Epidemiologic Surveillance, Europe, 2021. Emerg Infect Dis. 2021;27(10):2738-2739. https://doi.org/10.3201/eid2710.210704

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Subsequent "consensus" statement

Table 1. Key question about D/N_{752} strains

Is there a particular EHV-1 strain that causes neurological disease?	No DNA_{pol} (ORF30) variants carrying the D_{752} marker are associated with most neurological disease outbreaks
Are all outbreaks of neurological disease caused by D_{752} viruses?	No D_{752} viruses are more commonly isolated from horses that suffered from neurological disease than N_{752}
Are N_{752} viruses nonpathogenic?	No N_{752} viruses are isolated from the majority of abortion outbreaks and a minority of neurological disease outbreaks worldwide
What proportion of horses carry D_{752} viruses?	Not known Available data suggest that 5–20% of EHV-1 viruses have the D_{752} genotype, but this data has been generated from a small number of studies
Is D/N_{752} testing useful?	Debatable Knowing the D/N_{752} genotype of an EHV-1 isolate is not relevant to the prevention and control of EHV-1 abortion outbreaks If an EHV-1 outbreak is associated with neurological signs, strict disease control measures should be imposed regardless of D/N_{752} genotype If an active EHV-1 infection, as evidenced by viremia and/or shedding, is diagnosed as D_{752} positive, even in the absence of neurological signs, it is possible that there could be an increased risk of a neurological disease outbreak. However, no study to date has properly tested this relationship The most important reason to perform this testing is to increase our knowledge about EHV-1 epidemiology

J Vet Intern Med 2009;23:450–461

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Conclusions

- **A previous study on EHV-1 genotypes described strain variations and detected a point mutation at ORF 30, which was associated with the anamnestic report of neurologic symptoms**
- **Credo: there is (most likely) a direct correlation between pathogenicity of strains, the amount of abortions induced and the appearance of neurologic symptoms**
- ***"The results of our experimental infection studies demonstrate that the N752 sequence variant of EHV-1 DNA Pol, when compared to the D752 variant, has reduced overall pathogenic potential and capacity to induce neurological signs."***
- **The variant D752 was probably on the rise**
- **At present, however, there seems to be limited diagnostic value in the analysis of the ORF30 mutation, since it only describes the potential**
- **The effect of host factors must not be underestimated !**

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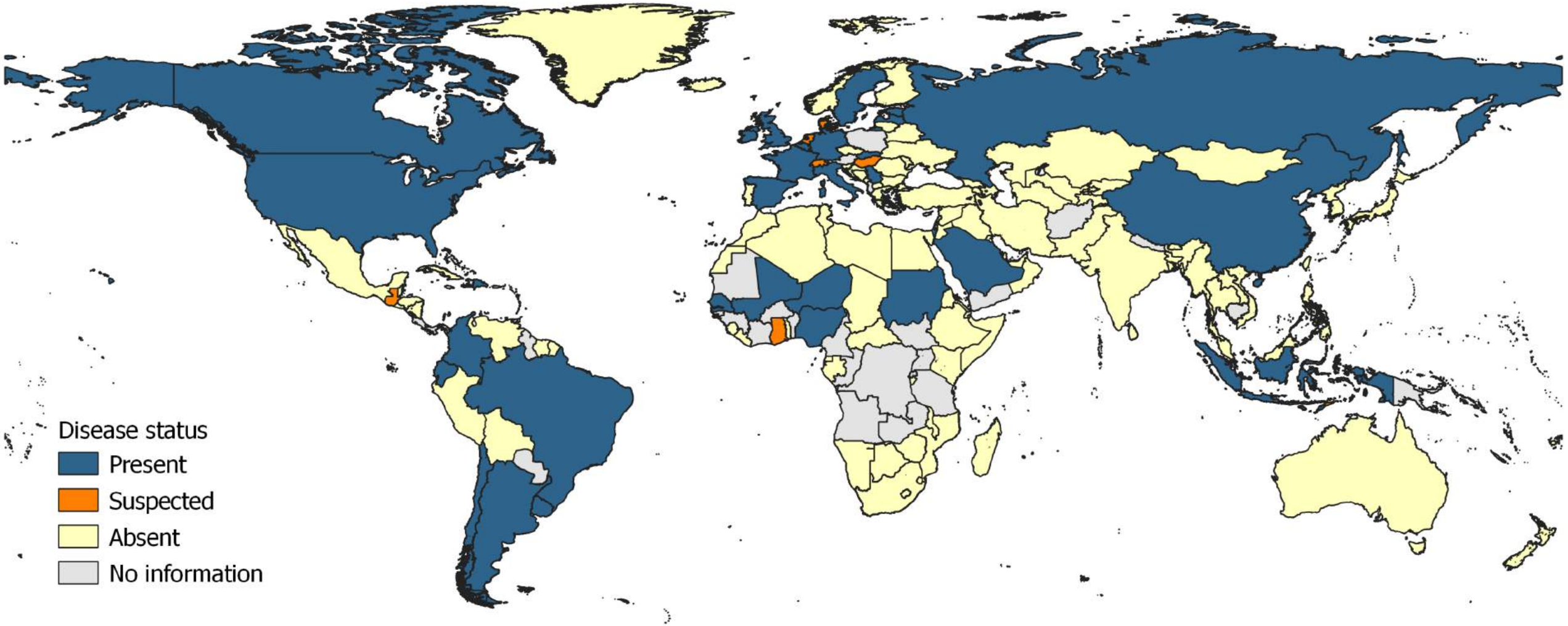
EIV 2016-2020



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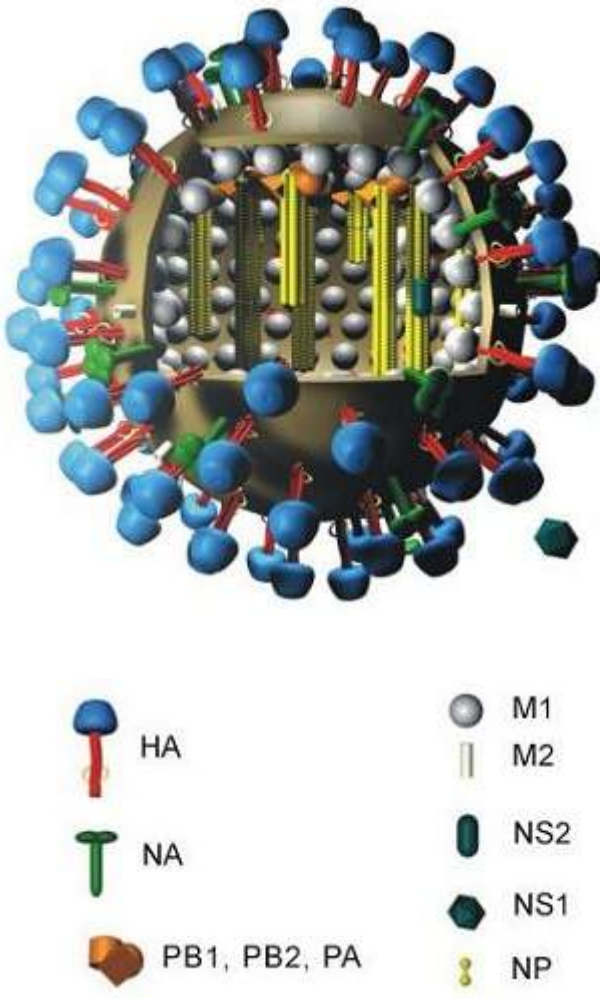
Structure

- Negative strand RNA segmented genome
- 8 gene segments, 10 genes
- On the surface are the two glycoproteins haemagglutinin (HA) and neuraminidase (NA)
- Two matrix proteins
- Two nonstructural proteins.
- Three proteins that make up the RNA polymerase
- Nucleoprotein



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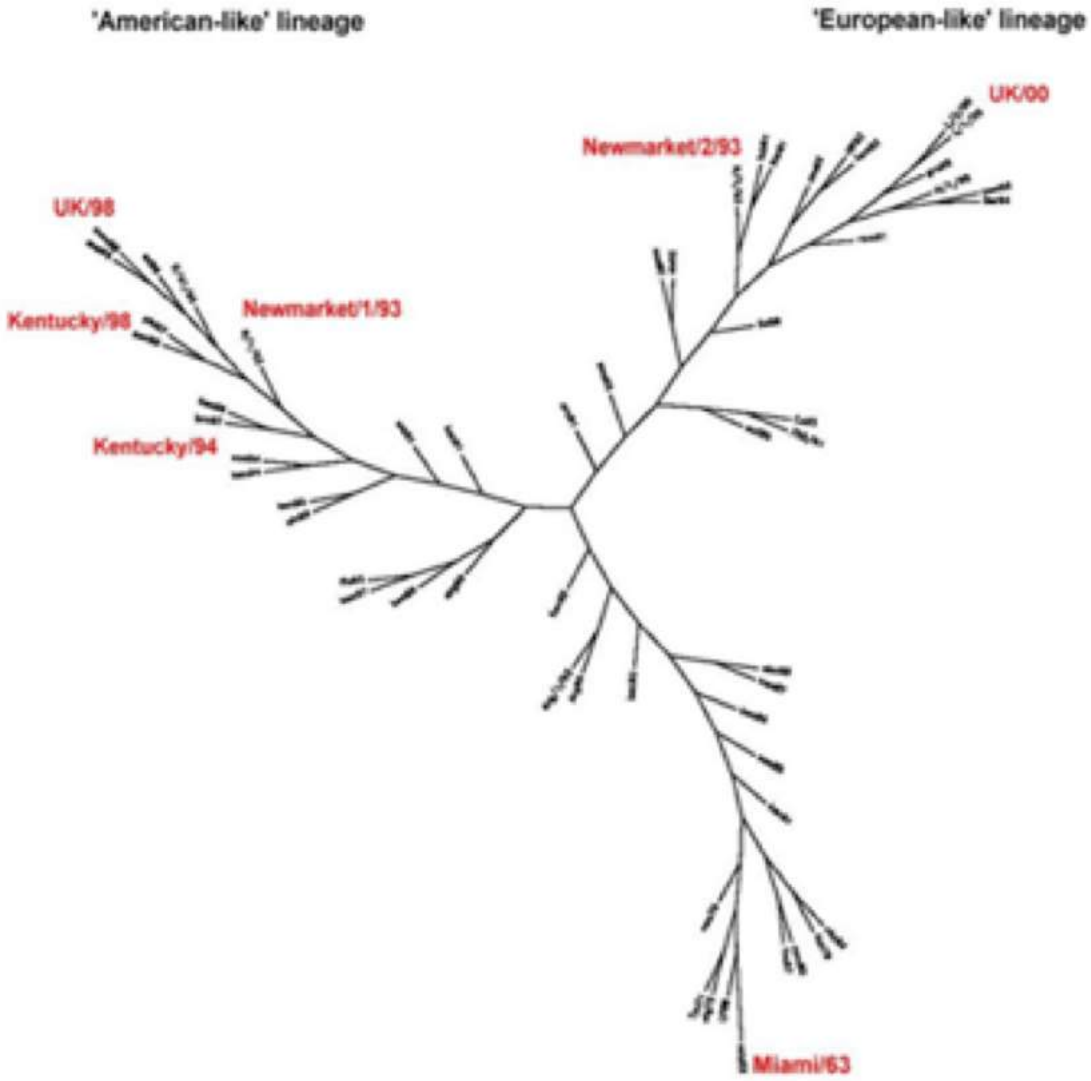
Phylogenetic tree for equine influenza H3N8 from 1963 to the present day

- Equine influenza viruses belong to the H7N7 and H3N8 subtypes, those currently circulating are H3N8.
- In the late 1980s the virus haemagglutinin sequences diverged into two separate branches, the American and the European sublineages.
- The American branch has further subdivided to form the ‘variant American’ sublineage and most of the viruses circulating in the UK belong to this branch. These are slightly different to those found in North America, which also belong to this branch.



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Transmission

- The disease spreads very rapidly (24-72h) to susceptible in-contact horses causing high morbidity.
- Transmission of influenza is frequently facilitated through a combination of vaccines successfully attenuating the clinical signs but failing to prevent viral shedding.
  - May be due to variable vaccine potency
  - Poor response to vaccination
  - Antigenic drift or a combination of these factors
- Equine influenza is important in the international movement of horses and is included on the OIE list of notifiable diseases.

Disease	Risk of undetected presence in the subpopulation	Risk of introduction into the subpopulation	Risk of transmission within and from subpopulation
EI	Yes* (Subclinical infection in partially immune horses)	Yes (Effective airborne transmission, risk increased during air transportation)	Yes (Pre-symptomatic shedding ; asympatomatic shedding in partially immune horses ; air-borne transmission not excluded within the subpopulation)

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Diagnostic Tests – RT-qPCR

- *Antigen in nasal secretions may be detected directly by an antigen-capture enzyme-linked immunosorbent assay (ELISA) employing a monoclonal antibody against the nucleoprotein. (Cook et al., 1988; Livesay et al., 1993).*
- A realtime time PCR assay for detection on matrix gene was described by Spackman *et al*. 2002.
- Tested for detection of all 16 HA types and NA types.
- Tested sensitivity and specificity against VI and HI which were comparable.
- This method is used in many labs including APHA for influenza detection

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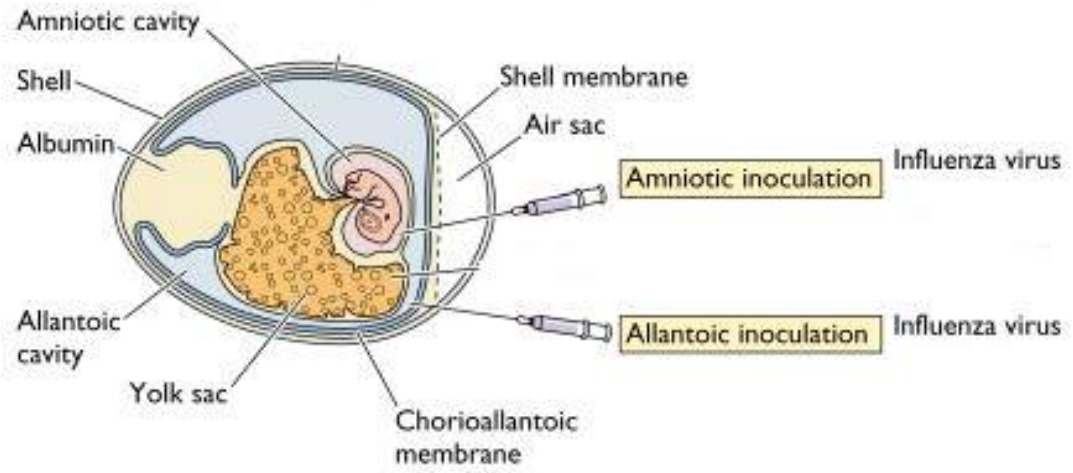
Diagnostic tests – virus isolation



- Traditionally equine influenza is diagnosed by the isolation of virus from nasopharyngeal swabs in embroynated hen eggs
- Former OIE 'gold standard':
- ……but may require up to several days for a result
- Need follow up via e.g. HI

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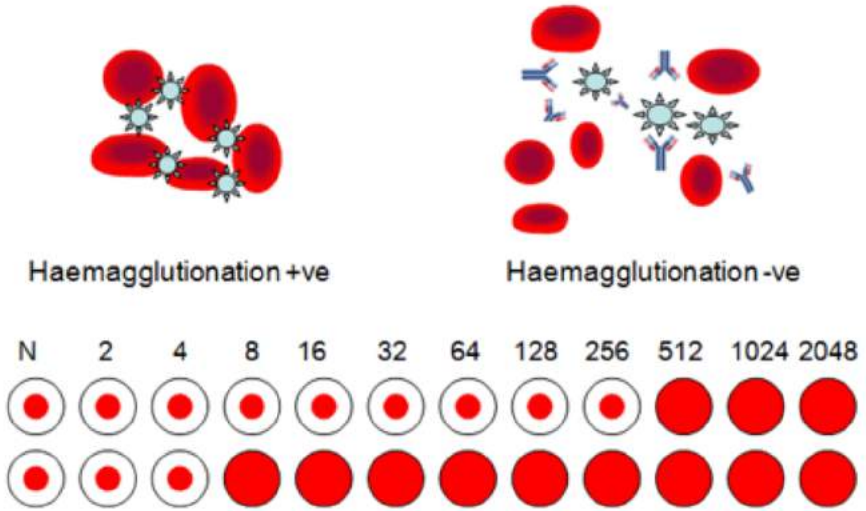
Diagnostic Tests – haemagglutination based

- The haemagglutination–inhibition (HI) test and is based on recognition of virus by a panel of reference antibodies.
- Influenza viruses bind to red blood cells using the haemagglutinin molecule and agglutinate them, a process which is easily seen if virus and red blood cells are mixed together in the correct proportions and plated out in a 96-well plate.
- By serially diluting specific antibodies and adding these to the virus it is possible to block this interaction and measure how closely the virus is related to the antisera and to previous strains.
- In the diagram below, the top row of wells shows a strong interaction between virus and antiserum (titre = 256), the bottom row shows a weak interaction (titre = 4)



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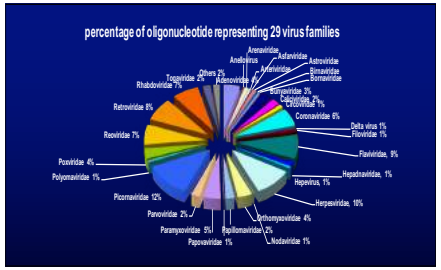
Summary

Methods to detect new and emerging viruses

Pan-virus specific PCRs

pan-Herpes
pan PestiV
pan Flavivirus
etc

Microarray



Next generation (aka "deep") Sequencing

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