EQUINE INFECTIOUS ANAEMIA:
LESSONS LEARNED FROM THE SIX-YEAR APPLICATION OF
THE NATIONAL ITALIAN SURVEILLANCE
Rome 1st October 2012

DYNAMICS OF EQUINE INFECTIOUS ANEMIA VIRUS
INFECTION IN NATURALLY INFECTED MULES

Study conducted within Research Project IZSLT 07/08 RC, approved and funded by the Italian Ministry of Health
Distribution of EIA cases among the species examined during the surveillance period 2007-2011

http://www.izs.it/izslt/

2007-2011 EIA surveillance activity report of the Italian National Reference Centre for Equine Diseases & Equine Infectious Anaemia
How to interpret the infection status of apparent false negative AGIDT reactions – predictive value of Elisa/IB positive reactions?

<table>
<thead>
<tr>
<th>Category</th>
<th>Number</th>
<th>Rate</th>
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<tbody>
<tr>
<td>Samples tested in survey</td>
<td>96,468</td>
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<tr>
<td>Positive in IT C-ELISA</td>
<td>331</td>
<td>0.34%</td>
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<tr>
<td>Positive IT C-ELISA and AGIDT</td>
<td>124</td>
<td>0.13%</td>
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<tr>
<td>Positive IT C-ELISA and Negative AGIDT</td>
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<tr>
<td>Negative Immunoblot</td>
<td>182</td>
<td>88%</td>
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<td>Positive Immunoblot</td>
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<td>12%</td>
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<td>Overall number judged positive for EIA</td>
<td>124+25 = 149</td>
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<td>Total Number of samples tested in survey</td>
<td>96,468</td>
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<tr>
<td>Apparent False-Positive IT C-ELISA</td>
<td>182</td>
<td>0.19%</td>
</tr>
<tr>
<td>Apparent False-Negative AGIDT</td>
<td>25</td>
<td>0.026%</td>
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</table>

13/25 of the apparent false negative AGIDT were mules with 11/13 coming from prevalent outbreaks.
Clinical signs of AIE reported especially in horses

The disease most frequently takes on a subclinical/inapparent course but can also be acute & chronic in horses infected with the same viral strain (Hammond et. al. 2000, Leroux et. al. 2001, Cook et. al. 2001).

Recurrences can be stress-related and may be provoked by malnutrition, overwork or surgery.

Chronic cases are characterised by intermittent cycles of: fever, anaemia, oedema, weight loss and lethargy alternated by periods of normality.

Enlarged grey red liver showing lobular pattern and haemorrhage under the capsule.
Eg. of principal parameters evaluated during EIAV (experimental) infections
Limited studies available in literature regarding the evolution of AIE infection in mules

Equine infectious anemia in mules: virus isolation and pathogenicity studies

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Objective of the study
Determine the **virological status & epidemiological significance** of mules with an EIA equivocal serological result

<table>
<thead>
<tr>
<th>Experimental Identification N°</th>
<th>Province of Origin</th>
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<tr>
<td>2</td>
<td>Latina</td>
<td>2</td>
<td>F</td>
</tr>
<tr>
<td>3</td>
<td>Aquila</td>
<td>30</td>
<td>F</td>
</tr>
<tr>
<td>4</td>
<td>Roma</td>
<td>22</td>
<td>F</td>
</tr>
<tr>
<td>5</td>
<td>Roma</td>
<td>9</td>
<td>F</td>
</tr>
<tr>
<td>6</td>
<td>Roma</td>
<td>8</td>
<td>F</td>
</tr>
<tr>
<td>7</td>
<td>Frosinone</td>
<td>11</td>
<td>CM</td>
</tr>
<tr>
<td>8</td>
<td>Aquila</td>
<td>17</td>
<td>F</td>
</tr>
<tr>
<td>9</td>
<td>Frosinone</td>
<td>11</td>
<td>F</td>
</tr>
<tr>
<td>10</td>
<td>Frosinone</td>
<td>7</td>
<td>F</td>
</tr>
</tbody>
</table>

Longitudinal study on **10 naturally infected mules from 5 different outbreaks**, including **5 subjects with equivocal Elisa/AGIDT serological patterns**

Details of study group & experimental set-up
Details of study group & experimental set-up

Clinical anamnesis referred by owners was that some of the animals had performance loss and weakness with no other apparent clinical.

The mules were purchased only after the owners had consented their slaughter.

The premises where the study carried out was authorised by the Regional & Local Veterinary Services.

During the study, the premises were declared as an outbreak for AIE & the biosecurity measures prescribed in the AIE Regulation were applied for the whole observation period.
Confirmation of EIAV infection on pre-enrolment samples by:

-all mules had positive PCR products which when sequenced were homologous with circulating strains of AIEV, *Cappelli et al*, 2011.

-all mules were confirmed as positive in immunoblot (IB), *Issel et. al*, 1999.
Experimental set-up

**Observation period**
Approx. four months.

**Clinical evaluation**
Monitored daily - general examination of each animal.

**Sample collection**
Blood with & without anti-coagulant were collected on daily basis.

**Immunosuppression (IS) & verification** - dexamethasone administration, halfway through observation period (*Craigo et al., 2007*), doses within accepted therapeutic levels.
Immune status was monitored pre and post IS through a **delayed-type hypersensitivity assay** (DTH) induced by PHA.

DTH ratios were calculated as the ratio of antigen (PHA) reaction to control (saline) reaction - the control reaction (saline alone) was divided into the PHA reaction to yield the DTH ratio (Y axis).
Laboratory parameters investigated & diagnostic methods employed

Platelet count (log\(_{10}/\mu L\)) was determined using an automated counter Cell-Dyn 3700 (ABBOTT)

Qualitative and quantitative humoral responses were investigated using the three tier system;

- in-house C-ELISA — (Amaddeo et. al, 1998)
- quantitative evaluation of the serological response

Results expressed as reciprocal of last dilution still reacting positive

- AGIDT — (Coggins et. al, 1972)
  OIE Diagnostic Terrestrial Manual, 2008

IB — (Issel et. al, 1999) — positive

if reacting to p26 and also to at least 1 of the other 2 gps
Plasma associated viral-RNA loads – determined by using a TaqMan® based RT-PCR, directed against exon1 of *tat* gene using a quantified internal standard for the determination of RNA viral copies

Primer design - by Dr. F. Cook  
**MkIII Forward**: 5′-GGC GCC CGA ACA GGG ACC-3’ (UK position numbers = 310-327)  
**MkIII Reverse 1**: 5′-TGG CCA GGA ACA CCT CCA GAA GAC-3’ (UK position numbers = 405-428)  
**Probe LNA EIAV**: 5’-FAM –T+GA ACC T+GG +CTG ATC G+TA G+GA-3’BHQ 1

**Reaction profiles**  
1<sup>st</sup> step – RNA extraction - starting from 140 µl of plasma, using the automatic extractor Qiacube® with the Qiagen Viral RNA Mini kit.  
2<sup>nd</sup> step – cDNA synthesis - High Capacity cDNA Archive Kit (Applied Biosystems®)  
Thermal profile - 25°C for 10’, 37°C for 120’, 85°C for 5’ & 4°C ∞  
3<sup>rd</sup> step - Real Time qPCR - TaqMan® Universal PCR Master Mix (Applied Biosystems®)  
Thermal profile - 50°C for 2’, 95°C for 10’, 50 cycles: 95°C for 15”, 52°C for 30” e 60°C for 1’, 72°C for 2’
### RESULTS

#### Reactivity of serum samples in AGIDT Mules

<table>
<thead>
<tr>
<th>Days Post-IS</th>
<th>10</th>
<th>2</th>
<th>4</th>
<th>9</th>
<th>1</th>
<th>5</th>
<th>8</th>
<th>7</th>
<th>3</th>
<th>6</th>
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<tbody>
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<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
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<td>1</td>
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<td>2</td>
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<td>3</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>1</td>
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<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>28</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>1</td>
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<td>0</td>
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#### Reciprocal of end-point Elisa titres

<table>
<thead>
<tr>
<th>Days Post-IS</th>
<th>4</th>
<th>10</th>
<th>9</th>
<th>2</th>
<th>1</th>
<th>5</th>
<th>8</th>
<th>7</th>
<th>3</th>
<th>6</th>
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<tbody>
<tr>
<td>-7</td>
<td>576</td>
<td>192</td>
<td>24</td>
<td>192</td>
<td>48</td>
<td>12</td>
<td>48</td>
<td>6</td>
<td>0</td>
<td>0</td>
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<tr>
<td>0</td>
<td>576</td>
<td>192</td>
<td>48</td>
<td>192</td>
<td>192</td>
<td>12</td>
<td>24</td>
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<td>7</td>
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<td>192</td>
<td>48</td>
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<td>48</td>
<td>12</td>
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<td>21</td>
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<td>28</td>
<td>192</td>
<td>576</td>
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<td>192</td>
<td>192</td>
<td>48</td>
<td>192</td>
<td>48</td>
<td>12</td>
<td>+/-</td>
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</tbody>
</table>

Higher reacting

Lower reacting

Ranked AGIDT & Elisa serological profiles
IB of mules with higher AGIDT/Elisa reactivity
IB of the mules with lower AGIDT/Elisa reactivity
Temporal profiles of plasma RNA viral load/plt/°C

Mule 1
results of viral rna load/ PLT/ °C

| days pre/post immunesupp. | -56 | -50 | -43 | -36 | -29 | -22 | -15 | -8  | -6  | 0  | 2  | 4  | 6  | 8  | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 24 | 26 | 28 |
|--------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| RNA viral load/ml        | -   | -   | -   | -   | -   | -   | -   | -   | -   | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| PLT/ul                   | -   | -   | -   | -   | -   | -   | -   | -   | -   | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| Baseline PLT/ul          | -   | -   | -   | -   | -   | -   | -   | -   | -   | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| Rectal Temp °C           | -   | -   | -   | -   | -   | -   | -   | -   | -   | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |
| Baseline °C              | -   | -   | -   | -   | -   | -   | -   | -   | -   | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |

TS
RNA viral load/plt/°C profiles of mules with higher AGIDT/Elisa reactivity
RNA viral load/ml
PLT/ul
Baseline PLT/ul
Rectal Temp
Baseline ° C

Mule 3
results of viral RNA load/PLT/° C

Mule 5
results of viral RNA load/PLT/° C

Mule 6
results of viral RNA load/PLT/° C

Mule 7
results of viral RNA load/PLT/° C

Mule 8
results of viral RNA load/PLT/° C

RNA viral load/ml
PLT/ul
Baseline PLT/ul
Rectal Temp
Baseline ° C

Mule 3
Mule 5
Mule 6
Mule 7
Mule 8

RNA viral load/ml
PLT/ul
Baseline PLT/ul
Rectal Temp
Baseline ° C

Mule 3 Mule 5
Mule 6 Mule 8

RNA viral load/plt/°C profiles of mules with lower AGIDT/Elisa reactivity
Clinical signs were mild to absent, probably mitigated because manifested during recurring episodes of the infection.
Other results

- Nucleotide sequencing confirmed that all mules were infected with EIAV - isolates similar to previously identified European strains (6 possessing close identity to EIAVRom-4 (GU060662.1), 2 to EIAVIta-1 (EU240733.1) and 2 to EIAVIta-90 (HQ888862.1)) - different pictures with same strain.

- Prior to IS, viral RNA was detectable during at least one sample point in 7 mules, including 1 with a negative AGIDT reaction (due to other stress conditions??????).

- In some of the animals, including the 3 with limited AGIDT reaction, there was extensive temporal variation in plasma-associated RNA viral loads - increase from 95 to 30,000 fold & significant differences between individual animals in the extent of viral replication with no guarantee that viral levels will be “limited”.
Other results – cont’d

• While all mules, following IS, showed increase in plasma-associated viral RNA loads, with values as high as $\log_{10}6$ RNA copies/ml for 4 subjects, only 3 had four-fold increases in C-ELISA titres & 2 remained AGIDT-negative (1 of which was also C-ELISA negative).

• No obvious correlation between viral RNA load response & negative serological results in AGIDT, individual characteristics & apparent viral strain.

• Similar viral loads have been observed in acute cases of AIE in horses in the Irish outbreak (Quinlivan M., et. al. 2007).
Other results – cont’d

• Only very mild transient clinical signs were observed for all mules & therefore the animals with negative/equivocal AGIDT reactivity would not have been captured by passive surveillance, solely based on clinical suspect.

• Subjects with low to null serological response in AGIDT cannot be considered as risk zero in the transmission of EIAV infection.
Only Considerations!

The exclusive use of the AGIDT could result in animals being falsely reported as negative and enhance the risk of diffusion?

One (AGIDT) or two tier (Elisa & AGIDT) system not enough!

High predicitive value of Elisa/IB positive result

Biosecurity measures prescribed for animals with equivocal AIE serological result should be as for those confirmed positive ...

What is the overall (direct and indirect) cost of these animals in a national surveillance programme?
Collaborations & acknowledgements

• Study performed in collaboration with the Gluck Equine Research Centre (Lexington, USA)
• Field support from the Veterinary Services of Roma G, Aquila, Latina
• Technical support from the staff of the IZSLT – Peripheral labs of LT and RI, & VIR, OEVIR, DIAG, RIA, DMV
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Vi ringrazio per l’attenzione

Thank you for your attention