PHARMACOLOGICAL REACTIVATION OF EQUINE INFECTIOUS ANAEMIA VIRUS IN NATURALLY INFECTED MULES: CLINICAL, HAEMATOLOGICAL AND SEROLOGICAL RESPONSES - Part 1

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SUMMARY

Equine Infectious Anaemia (EIA) is a viral vector borne disease of equids, including mules. In Italy, a high seroprelavence of this infection was observed in the mule population. In view of this situation and because of the scare information in literature relative to the infection in these animals, a study was conducted to evaluate the clinical, haematological and serological response of immune suppressed EIA infection in eleven naturally infected mules. The pharmacological immune suppression resulted in fever and/ or thrombocytopenia of eight mules. Of these, four mules also registered an increase of the serological response to the EIA virus. No direct correlation was observed between the clinical and haematological response and the serological reactivity. This study is the first report of the pharmacological reactivation of the EIA infection carried out to investigate the epidemiological role of these animals in the persistence and spread of EIA.

INTRODUCTION

EIA is a viral vector borne disease of equids even if epidemics have been traced to the multiple use of hypodermic needles and to the injection of substances contaminated with blood.
In Italy, since 2007 an extraordinary surveillance programme for the control of EIA was implemented due to a series of important outbreaks which had occurred over a short period in the spring of 2006. The programme imposes the serological control of all horses, donkeys and mules apart those reared for human consumption. During each of the three annual campaigns till now held, a marked higher seroprevalence was observed in mules, even if the numeric consistency of this population was conspicuously inferior when compared to that of the donkey and horse population. The numerous cases of EIA registered in these animals have until now never been reported in surveillance programmes held even in other countries. In view of this and also due to the limited literature available regarding EIA in mules, epidemiological, etiological and clinical studies of the infection in these animals have been undertaken to better understand their role in the persistence and spread of this infection.
In particular, this study reports the clinical, haematological and serological evolution of EIA in naturally infected mules following pharmacological reactivation.

MATERIALS AND METHODS

Experimental Animals: 11 EIA seropositive mules were recruited for the study coming from the provinces of Rieti, Rome, Frosinone, Latina and Aquila. Although the animals apparently came from independent outbreaks, the geographic localisation of these animals was in a relatively restricted area, represented by the dorsal area of the pre-Apennines of Central Italy.
The mules were identified with consecutive numbers from 1 to 11. The age of the animals ranged widely, with mules 2 and 11, between the age of 1 and 2 years, mules 1, 5, 6, 7, 9 and 10 between the age of 8 and 13 years, mule 8, of the age of 17 years and the mules 4 and 3, 22 and 30 years respectively.
All the animals were housed together and kept and feed under the same conditions for the whole experiment. Procedures of animal handling and experimentation were performed under veterinary supervision, according to the present European Regulations on Animal Experimentation.
Prior to the pharmacological reactivation of the EIA infection, the mules were treated for infections which could have interfered with the outcome of the EIA reactivation. The animals were vaccinated against EHV 1 and 4, treated for piroplasmosis and dewormed for intestinal parasites.
The animals were also reconfirmed as seropositive for the EIA virus (EIAV) before the start of the experiment in at least the following serological methods in C - Elisa and immunoblot (IB) described further on.
Procedures for immune suppression were based on protocols described by Craigo J.K. *et al.*, (2). Briefly, dexamethasone was administered intramuscularly for 8 days at a dose of 0.11mg/kg body weight/day for all except for 2 mules for which administration was extended to day 12. The mules were monitored daily for adverse reactions to treatment.
Skin tests for delayed-type hypersensitivity (DTH) reactions were performed during the pre-immune suppression and post immune suppression (P.IS.) periods and consisted in shaving and cleaning small areas on the neck and inoculating at different sites on the necks both 50µg of phytohemagglutinin (PHA) in 1ml of saline and 1ml of saline alone. The net increase in skin thickness was determined from measurements made with constant tension callipers 24 hours post-injection of antigen. DTH ratios were calculated as the ratio of antigen (PHA) reaction to control (saline) reaction.
The clinical signs for which the animals were controlled during the whole observation period, starting from 7 days prior to the 1st day of cortisone treatment (day 0) to 28 days later, were those described by Leroux C. J. *et. al*. (2004) as those typically occurring during an EIA infection in horses: rise in body temperature, thromocytopenia. Other clinical signs for which the mules were monitored were alteration of their general condition, oedema, anaemia and congestion of the ocular and buccal mucosa, petechiae and jaundice. To avoid bias of registration of clinical signs by having the same operator for the P.IS. period.
Rectal temperatures were constantly measured at the same hour of the day and for these animals, a rectal above 39°C was considered as fever.
Biological samples for haematological and serological analyses, represented by blood, with and without anticoagulant, were collected daily for the whole experimental observation period.
Blood samples, collected throughout the whole experimental period, from the jugular vein in tubes with and without K3-EDTA, were refrigerated at 4°C and immediately sent to the laboratory. An automated counter Cell-Dyn 3700 (ABBOTT) was used to determine the platelet (PLT) counts. Baseline value for PLT was fixed at 100 x 10^3/µl.
Serum was obtained from blood samples after centrifugation for 10 minutes at 179 **g**, the supernatant was then collected and stored at –20°C.
The serological methods used in the study were the following:

an in-house C-Elisa, the Agar Gel Immunodiffusion Test (Agid) and the Immunoblotting (IB). The reason for employing these tests was that they are the serological methods contemplated in the EIA surveillance programme and therefore used to evaluate possible difference in reactivity for each method through the course of the trial.

The methods are briefly described as following.

The in-house C-Elisa used was developed in collaboration with the Istituto Zooprofilattico Sperimentale delle Regioni Lombardia ed Emilia Romagna. The method consists in sensitising Nunc Maxisorp ® plates overnight at 4° C, using as catcher an anti-p26 monoclonal antibody (Mab) diluted in phosphate buffer solution. In time for the end of the sensitisation, the serum samples are prepared by diluting them from 1/3 to 1/192 on inert microplates in PBS pH 7,2 –7,4, containing yeast extract (0.05%) and mouse serum (1%) together with the following internal controls: an antigen control, a positive and negative control and a blank reaction control. The recombinant p26 antigen is added to the samples and controls, all of which are examined in double replicates, so that the final dilutions obtained are the double those mentioned earlier (from 1/6 to 1/384). At the end of a 75' incubation at 37°C, the samples and controls are transferred onto the previously washed sensitised plate. On terminating the distribution of the samples, the horseradish conjugated tracer Mab is added, so as to then proceed with another incubation under the same conditions as before. The reaction is developed by the addition of OPD substrate and stopped after 10', using 1M sulphuric acid. The samples reactivity is read at 492 nm using a spectrophotometer.

The results are interpreted using the following algorithm:

Percentage Inhibition (PI) = 100 - (OD mean of sample/OD mean of negative control X 100). The end–point titre of a serum was the reciprocal of the dilution which was still below 50% inhibition.

The Agid was carried out using the method described by the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2010 as well as that described by Coggins (5). The antigen used for both methods is the recombinant p26, produced by our Institute (1). After 24 – 48 hours the plates are examined for the precipitation line between the sample the antigen well, typical of a positive serological reaction in Agid.

The IB was conducted as described by Issel C. J. *et. al.,* (4), diluting 1/50 the negative and positive controls and the samples, or 1/80 if the reactivity of the serum was still too strong. The reagents, as well as the internal controls, were supplied by the same authors. The samples were examined for their reactivity for the capsid protein p26 and for the transmembrane (gp45) and the surface (gp 90) glycoproteins.
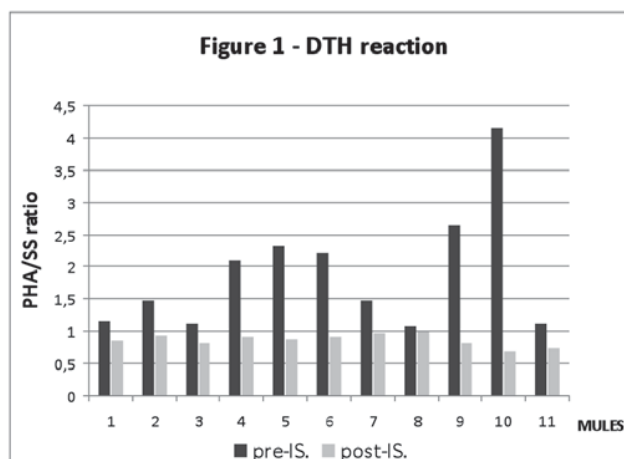
For all the serological methods, the samples of each mule were examined all together on the same day, as also on the same plate for the C- Elisa and on the same membrane for the IB to avoid possible variability arising from examining the samples at different times.

An EHV 1 and 4 duplex Real Time PCR as decribed by Damiani A. *et. al.,* (4) was carried out on the nasal swabs collected during the P.IS. period to verify the reactivation of these possible latent Herpesvirus infections.

Results - Part 1

Verification of immune suppression (Figure 1): prior to immune suppression, DTH tests were performed to establish baseline levels of immune reactivity. The DTH assays indicated as also observed by other authors (2) .an effective suppression of host immunity by day 8 for all mules except for mule 1 and 11 which was reached by day 12 of the drug treatment.

Remaining results and discussion are presented in Part- 2.

Figure 1 - DTH reaction

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