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Evolution of equine infectious anaemia in naturally infected mules with different serological reactivity patterns prior and after immune suppression

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ABSTRACT

Information on equine infectious anaemia (EIA) in mules, including those with an equivocal reaction in agar gel immunodiffusion test (AGIDT), is scarce. For this, a study was conducted to evaluate the clinical, viral loads and pathological findings of two groups of naturally infected asymptomatic mules, respectively with a negative/equivocal and positive AGIDT reactivity, which were subjected to pharmacological immune suppression (IS). A non-infected control was included in the study that remained negative during the observation period. Throughout the whole study, even repeated episodes of recrudescence of EIA were observed in 9 infected mules, independently from their AGIDT reactivity. These events were generally characterised by mild, transient alterations, typical of the EIA acute form represented by hyperthermia and thrombocytopenia, in concomitance with viral RNA (vRNA) peaks that were higher in the Post-IS period, reaching values similar to those of horses during the clinical acute phase of EIA. Total tissue viral nucleic acid loads were greatest in animals with the major vRNA activity and in particular in those with negative/equivocal AGIDT reactivity. vRNA replication levels were around 10–1000 times lower than those reported in horses, with the animals still presenting typical alterations of EIA reactivation. Macroscopic lesions were absent in all the infected animals while histological alterations were characterised by lymphomonocyte infiltrates and moderate hemosiderosis in the cytoplasm of macrophages. On the basis of the above results, even mules with an equivocal/negative AGIDT reaction may act as EIAV reservoirs. Moreover, such animals could escape detection due to the low AGIDT sensitivity and therefore contribute to the maintenance and spread of the infection.

1. Introduction

Equine infectious anaemia (EIA) is a viral disease that affects all domestic members of the genus Equus spp. (horse, donkey and mule) and is characterised by intermittent fever, progressive anaemia, emaciation and death in severe cases. Clinical forms of EIA are acute, chronic and inapparent, with the latter being the most frequent. Animals in the chronic and inapparent phase may experience a recrudescence of the infection as a consequence of severe stress, hard work or the presence of other diseases (Quinlivan et al., 2007). EIA virus (EIAV), a Lentivirus of the Retroviridae family, is responsible for the infection that is characterised by a restricted tropism for the equine monocyte-macrophage lineage with productive replication occurring only in the differentiated tissue macrophages (Oaks et al., 1998).

During the surveillance programme conducted in Italy, between 2007 and 2012, that used the ELISA as a screening test and agar gel immunodiffusion test (AGIDT) as a confirmatory test, prevalence in mules was found to be significantly higher than in horses and donkeys (Sala et al., 2012). In addition, equivocal AGIDT reactions were more frequent among this hybrid species (Scicluna et al., 2013).

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Studies on the carrier status with negative and equivocal AGIDT reactions are respectively described for horses, by McConnell and Katada, (1981) and by Issel and Adams (1982), in which the authors succeeded in the transmission of EIAV from such animals. Similar investigations were also conducted to evaluate the correlation of the AGIDT serological reactivity pattern with plasma viral loads at different time intervals and in the tissues of experimentally infected horses (Harrold et al., 2000; Hammond et al., 2000). In addition, in a study conducted by Cook et al. (2003), the authors report that the acute form of the disease in the horse is characterised by the typical EIA alterations of hyperthermia and thrombocytopenia which are observed when the plasma viral RNA (vRNA) copies exceed concentrations of $\log_{10}$ 7.7/ml, defined as the pathogenic threshold (Cook et al., 2001). Different was the evolution observed in donkeys as these animals maintained low viremic levels in the absence of clinical signs (Cook et al., 2001).

Clinical and virolgical studies on EIA in mules are limited to two publications, in which the authors, in one of them (Spyrou et al., 2003), describe in detail the evolution of the natural and experimental infection observed in two subjects for each condition, for a time period that was however insufficient to completely elucidate this. Also, the presence of EIAV in these animals was reported only qualitatively. Considering the relatively high EIA prevalence levels in the Italian mule population and their elevated density in restricted areas where they are used as working animals, the scientific literature on the importance of the role that they might play as EIAV reservoirs is to date scarce. In view of this, an observational study was conducted to assess the clinical, pathological and virological parameters of naturally EIA infected mules having equivocal/negative AGIDT reactions, according to the scoring system proposed by Issel et al. (1999). In addition, the results obtained from these animals were compared to those having a clearly positive AGIDT reaction. As all the study animals were presenting an inapparent form of the infection, they were subjected to a pharmacological immune suppression (IS) as described by Craigo et al. (2007) for the reactivation of the disease. Further to the serological findings, already described by Scicluna et al. (2013), this paper reports the clinical evolution of EIA, in relation to the plasma vRNA levels, with the aim to investigate the correlations among these characteristics and evaluate the results in relation to the risk such animals may represent in the epidemiology of EIA. Viral replication was assessed in terms of vRNA copies in plasma, as well as, total viral nucleic acid loads (viral DNA and RNA) and presence of gross and histological lesions in tissues of various organs collected at the end of the study period (SP).

2. Materials and methods

2.1. Experimental animals and design

Ten mules, arbitrarily identified as they were enrolled from 1 to 10, were acquired from five distinct EIA outbreaks that had occurred in five neighbouring provinces of Central Italy. The age, sex and serological status for EIAV, of the experimental animals recorded on recruitment, are reported in Table 1. The ten animals were divided into two groups on the basis of their AGIDT reactivity observed at the start of the experiment: five mules had either equivocal or negative reactions (Group N) while the remaining animals presented a clear precipitation identity band (Group P), (Scicluna et al., 2013). A negative control, Mule 11, was included in the study that was however kept isolated from the infected animals, but subjected to the same experimental conditions adopted for the other ten mules. Clinical examination and sampling of the negative control were carried out with the same frequency and using the same methods as those described for the infected animals. To ensure its persistent negative status, the EIAV tests on this animal were extended to 30 days after the end of the SP.

The study design was approved by the competent authorities (Italian Ministry of Health – identification No IZS 02/10 RC). Animal husbandry and experimental procedures adopted throughout the SP, that lasted for a minimum of 84 days, were conducted under veterinary supervision and in compliance with the European Union Regulations in force for the use of animals in experiments. Biosecurity measures, as prescribed by the National Regulations, were also adopted to ensure that no EIAV transmission occurred from these animals.

2.2. Pharmacologically induced immune suppression

The mules included in the study underwent IS to induce EIAV reactivation. The treatment protocol, described by Kono et al. (1976) and Tumas et al. (1994), started on day 56 from the start of the SP using dexamethasone (Rapison®) at 0.11 mg/kg body weight/die. The duration of the pharmacological administration for each animal was based on the Delayed Type Hypersensitivity (DTH) reaction obtained by the inoculation of *Phaseolus vulgaris* agglutinin® (PHA – Sigma) with the same procedure described by Baus et al. (1996).

2.3. Clinical examination

The general condition of the animals, including the clinical signs considered as characteristic of an acute form of EIA,

Table 1
Details of the characteristics of the study group: identification, age, gender, serological results observed on recruitment and at the end of OP.

Mule identification no.	Age	Gender	Serological results (ELISA/AGIDT/IB[a])		AGIDT reactivity groups[b]	Duration of IS treatment in days
			On recruitment	At the end of SP		
1	12	F	+/2/+	+/2/+	P	10
2	2	F	+/3/+	+/3/+	P	8
3	30	F	+/0/+	+/0/+	N	8
4	22	F	+/3/+	+/3/+	P	8
5	9	F	+/1/+	+/2/+	N	8
6	8	F	±/0/+	−/0/+	N	8
7	11	C	+/1/+	+/1/+	N	8
8	17	F	+/1/+	+/1/+	N	8
9	11	F	+/2/+	+/4/+	P	8
10	7	F	+/3/+	+/4/+	P	8

[a] Serological results for ELISA and IB are reported qualitatively as positive (+) or negative (−). The AGIDT reactivity is reported as a score as described by Issel et al. (1999).
[b] AGIDT reactivity group: N mules with negative (score 0) or equivocal (score 1) reactivity and P mules with clear reactivity (score 2–5).

represented by hyperthermia, diarrhoea, lethargy and anaemia (Leroux et al., 2004), were blindly recorded during a complete physical examination, conducted daily for the whole SP, at the same hour by one clinician to reduce variability in their registration. Threshold for hyperthermia was set at a rectal temperature above 39 °C as reported for horses in similar studies, (Issel et al., 2014).

2.4. Sample collection for laboratory examinations

During the daily clinical examination of the animals, blood, with and without EDTA, were collected from each animal for the laboratory tests described in the present paper. For the estimation of vRNA, plasma was obtained from EDTA blood as follows; volumes of 5 ml of blood were processed on collection by centrifugation at 500×*g* for 5 minutes (min) to separate the plasma from the erythrocytes. The plasma was transferred into a new tube, centrifuged at 1200×*g* for 30 min and the resulting plasma was collected and stored. Nasal swabbing was performed to verify active infections due to equine herpesvirus 1 and 4 and to investigate the possible elimination of EIAV through this route in relation to the presence of the virus in the lungs of affected horses as described by Quinlivan et al. (2007) and Bolfa et al. (2013). On collection, the swabs were immediately immersed in 0.5 ml of phosphate buffer solution (PBS). The supernatant of these samples was collected after centrifugation for 20 min at 1000×*g*. Blood serum was obtained by centrifugation for 10 min at 179×*g*. At the end of the SP, the animals were slaughtered according to the Regulations in force and subjected to necropsy. Tissue samples of the following organs were collected: brain, spinal cord, spleen, lung, heart, mediastinal and mesenteric lymph nodes, liver, kidney and adrenal gland. Samples for virological and serological tests were stored at −80 °C and −20 °C respectively until further examination, and in the case of histological and immunohistochemical analyses, organ samples were processed as described further on.

2.5. Serological and biomolecular methods for pathogens other than EIA

On recruiting, the animals were verified for the presence of infections that could cause a similar clinical picture or influence the evolution of the EIA infection during the study period. The infections considered were based on the local epidemiological situation and clinical pictures similar to EIA that these could induce, as in the case of equine piroplasmosis (EP) caused by *T. equi* and *B. caballi* or immune suppression due to equine herpes virus (EHV) 1 and 4. Testing for presence of these etiological agents was carried out as follows: the serum of the experimental animals was tested for antibodies against equine piroplasms using Babesia equi/caballi Antibody test Kits, cELISA–VMRD, Inc.®. EDTA blood samples were used for the molecular detection of the two protozoa, employing commercial PCR kits, Babesia equi/caballi Genesig® Advanced (PrimerdesignTM Ltd, Southampton, United Kingdom). Animals positive for EP were treated with Imidocarb® (Intervet), a week before the start of the SP, with the dosage indicated for the sterilization of the two protozoa. For EHV 1 and 4, vaccination using Pneumabort K® (Fort Dodge Laboratories) was administered three weeks prior to the start of the SP, followed by a booster four weeks later.

The efficacy of the EP treatment was verified by retesting the subjects using PCR, two weeks after treatment and again weekly throughout the whole SP. Presence of EHV 1 and 4 infections were also monitored on weekly basis, using a Real Time PCR (RT PCR) as described by Damiani et al. (2005).

2.6. Bacteriological examinations

For this purpose, nasal swabs were collected and immediately immersed in Stuart's transport medium and stored at −20 °C for further use. Bacteriological examinations were carried out only if an animal presented clinical signs referable to a respiratory infection of bacterial origin.

2.7. Haematological and PCR methods for EIA

2.7.1. Platelet count (PLT)

The PLT count was performed on EDTA blood, within an hour of its collection, using an automated Cell-Dyn 3700® (Abbott). As no reference baseline for this parameter is available in mules, the value adopted was that reported for horses at 1×10^5 platelets/μl (Issel et al., 2014). All the study animals had pre-SP values above the established baseline during the one-week observation period preceding the start of the experiment (data not shown).

2.7.2. Plasma vRNA loads

The evolution of EIAV replication in the mules was assessed as reported by Cook et al. (2003) and Quinlivan et al. (2007) through the quantitation of the vRNA in the plasma, collected on daily basis. vRNA levels were defined using a qRT PCR as described by Scicluna et al., 2013; targeting a region of the exon 1 of the tat gene of EIAV.

Total RNA was extracted in double replicate from a volume of 140 μl of each plasma sample using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) and employing the automatic extractor QIAcube (Qiagen, Hilden, Germany). Reverse transcription was conducted employing High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) on 200 ng of the extracted RNA, concentration established using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, U.S.A.). The total reaction volume was 60 μl, of which 30 μl contained 200 ng of the extracted RNA, with the addition of the following reagents: 6 μl of 10× random hexamer primers, 6 μl 10X RT-Buffer, 2.4 μl dNTP mix 100 mM, 3 μl of 5 U Multi Scribe Reverse Transcriptase and 12.6 μl of H_2O-DEPC. Synthesis of the cDNA was carried out using AB Veriti 96 well Thermal Cycler (Applied Biosystems, Carlsbad, USA) with the following thermal profile: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min.

The exon 1 of the tat gene of EIAV was used as target of the following primers and probe: MkIII Forward: 5′-GGC GCC CGA ACA GGG ACC-3′. MkIII Reverse: 5′-TGG CCA GGA ACA CCT CCA GAA GAC-3′. The probe employed is a Fluorescent Locked Nucleic Acid (LNA): 5′-FAM-T[+G]A ACC T[+G]G [+C]TG ATC G[+T]A G[+G]A-3′ BHQ. The qRT-PCR, was carried out using the TaqMan Universal PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) with the following composition: 12.5 μl of TaqMan 2X Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 900 nM of each primer, 300 nM of probe, 5 μl of cDNA and H_2O-DEPC to reach a total volume of 25 μl. The thermal profiles of the qRT-PCR, using AB 7900HT Fast Real-Time PCR System, were as following: 95 °C for 10 min, and 50 cycles of 95 °C for 15 s, 52 °C for 30 s and 60 °C for 1 min. A quantified control, developed as described further on, was included in each qRT-PCR run for the estimation of the vRNA copies/ml of the plasma sample. All data were analysed using the ABI 7900HT Sequence Detection Systems software package ver. 2.4 (Applied Biosystems, Foster City, CA, USA).

The limit of detection (LOD) of the assay was established using the *in vitro* transcribed RNA, obtained from the plasma of Mule 4 using the EIAV MkIII primers. Different Log_{10} concentrations of the transcribed RNA were prepared to cover a range between 3.77×10^9 to 0.2 copies/μl. The limit of quantitation (LOQ) of the qRT-PCR was defined as 3.4 copies/ml while the LOD was 1 copy/ml. The plasma vRNA load for each sample was determined by

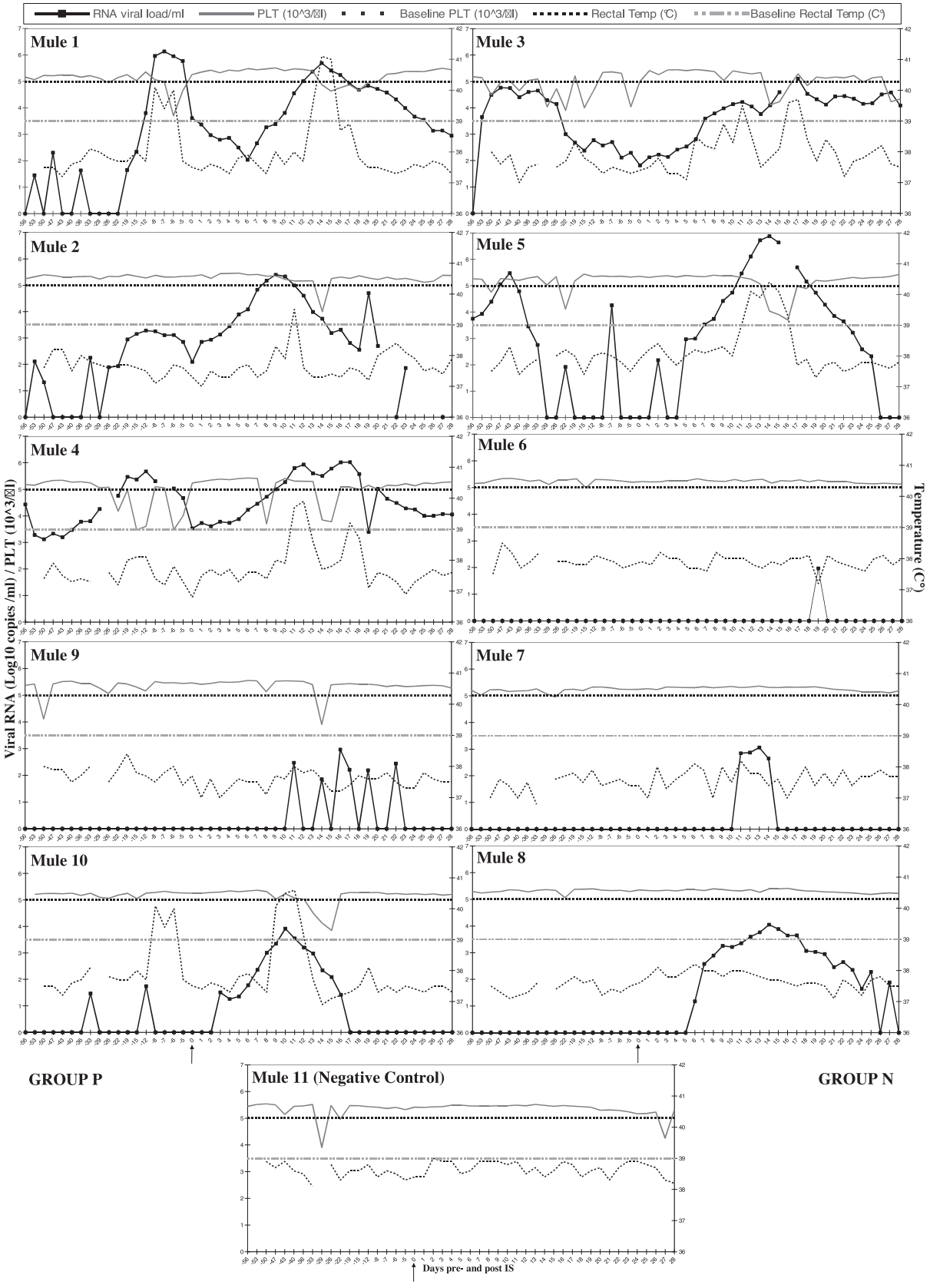

Fig. 1. Temporal evolution of body temperature, platelet counts and plasma vRNA load of study animals.

interpolation of the Ct value on the standard curve and reported as vRNA copies/ml. The Ct value of each plasma sample reported in Fig. 1 represents the mean value obtained from the analyses of the sample extracted and examined as a double replicate.

2.7.3. Total viral nucleic acid in tissues

For the quantitation of the total viral nucleic acid levels, that is vRNA and vDNA in the tissues collected at slaughter, 100 mg of each tissue type were added to 1 ml of PBS and disrupted using FastPrep® FP120Cell Disrupter Instrument (Qbiogene, Inc. Europe) in Lysing Matrix D tubes (MP Biomedicals, Santa Ana, California, USA). Two hundred μl of the homogenized tissue were employed for nucleic acid extraction with the automatic extractor QIAcube (Qiagen, Hilden, Germany) using the QIAamp cador Pathogen Mini Kit according to the manufacturer instructions. Each sample was extracted in double and the average nucleic acid concentration was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, U.S.A.). The average number of cells contained in each sample was estimated from the ratio of the mean value of the nucleic acid concentration of the sample and the average cellular content of nucleic acids, using the reference value reported on www.genomesize.com. This was carried out as previously described for the horse by Harrold et al. (2000) and in our case the assumptions used were that cells are diploid and in the Gl phase of the cell cycle (Simmonds et al., 1990). In addition, we assumed that the average cellular content of nucleic acids for the leucocytes of mules, the only data available, is equal to that contained in other tissues. The method used for the quantitation of the total viral nucleic acid loads in tissues was as described for vRNA.

The standardization of the nucleic acid viral loads in tissues was obtained by reporting the quantitative PCR results as the number of viral copies/100,000 cellular equivalent (10^5 CE).

2.7.4. Verification of the presence of PCR inhibiting activity

Presence of inhibiting activity in the samples examined by the PCR methods used was verified by the amplification of the endogenous gene coding for Beta-actin, using primers and probe included in the PCR kit of Babesia equi/caballi Genesig® Advanced (PrimerdesignTM Ltd, Southampton, United Kingdom).

Table 2
Histological, preliminary immunohistochemical (IHC) results and total nucleic acid viral loads in the tissues examined, divided, according to AGIDT reactivity, in Group P (positive), upper half of the table, and Group N (equivocal or negative), lower half.

GROUP P	Mule No 1 (49)				Mule No 2 (70)				Mule No 4 (49)				Mule No 9 (35)				Mule No 10 (35)			
	HISTOLOGY		IHC	Viral Load (copies/ 10^5 CE)	HISTOLOGY		IHC	Viral Load (copies/ 10^5 CE)	HISTOLOGY		IHC	Viral Load (copies/ 10^5 CE)	HISTOLOGY		IHC	Viral Load (copies/ 10^5 CE)	HISTOLOGY		IHC	Viral Load (copies/ 10^5 CE)
	HS	LI			HS	LI			HS	LI			HS	LI			HS	LI		
Adrenal Glands	na	na	na	–	na	na	na	–	–	–	/	1.16	–	–	/	–	–	–	+	1.73
Brain	–	+	/	–	–	–	/	< LOQ	na	na	na	–	–	–	/	–	–	–	/	–
Heart	–	+	/	–	–	–	/	–	–	+	/	0.83	–	–	/	–	–	+	+	–
Kidney	–	+	+	6.95	–	++	/	0.20	–	++	+	–	–	++	/	–	–	+	+	60.83
Liver	+	–	+	5.93	–	–	/	–	–	++	+	14.35	na	na	na	–	–	–	/	3.34
Lung	na	na	na	/	–	+	/	0.47	na	na	na	/	na	na	na	na	na	na	na	/
Mediastinal Lymph Nodes	–	–	/	–	–	–	/	–	–	–	/	1.37	–	–	/	–	–	–	/	–
Mesenteric Lymph Nodes	na	na	na	/	–	–	/	/	na	na	na	/	na	na	na	–	–	–	/	15.54
Spinal Cord	–	–	/	1.79	–	–	/	8.83	na	na	na	11.69	na	na	na	–	–	–	/	–
Spleen	++	–	/	25.19	–	–	/	21.92	++	–	/	0.17	++	–	/	46.86	++	–	–	4.44

GROUP N	Mule No 3 (28)				Mule No 5 (35)				Mule No 6 (70)				Mule No 7 (70)				Mule No 8 (56)			
	HISTOLOGY		IHC	Viral Load (copies/ 10^5 CE)	HISTOLOGY		IHC	Viral Load (copies/ 10^5 CE)	HISTOLOGY		IHC	Viral Load (copies/ 10^5 CE)	HISTOLOGY		IHC	Viral Load (copies/ 10^5 CE)	HISTOLOGY		IHC	Viral Load (copies/ 10^5 CE)
	HS	LI			HS	LI			HS	LI			HS	LI			HS	LI		
Adrenal Glands	na	na	na	na	–	–	/	4.69	–	+	+	–	na	na	na	–	na	na	na	–
Brain	–	++	?	50.99	na	na	na	0.15	–	+	/	–	–	–	/	–	–	–	/	–
Heart	–	+	/	–	–	+	/	0.08	–	+	/	–	–	+	/	–	–	–	/	–
Kidney	na	na	na	/	–	++	/	0.63	–	+	/	–	–	+	/	–	–	+	/	–
Liver	+	++	+	50.1	–	–	/	29.76	+	–	+	–	–	–	/	0.12	–	–	/	0.22
Lung	–	+	+	–	–	–	+	/	–	–	+	/	–	+	+	0.37	–	–	/	–
Mediastinal Lymph Nodes	+	–	/	6.23	–	–	/	2.51	–	–	–	–	–	–	/	1.3	++	–	/	–
Mesenteric Lymph Nodes	na	na	na	/	–	–	/	9.86	–	–	–	/	–	–	/	/	na	na	na	0.07
Spinal Cord	na	na	na	254.94	na	na	na	–	–	–	/	–	–	–	/	–	–	–	/	–
Spleen	+	–	/	129.25	++	–	/	487	++	–	–	–	+	–	/	40.34	++	–	/	–

Histopathological results are reported with the following score system: '-' no lesions, '+' mild (few and scattered lesions), '++' moderate to diffuse (multifocal to disseminated lesions); HS = hemosiderosis, LI = lymphomonocytic infiltrate. Total nucleic acid viral load are reported as no copies/10^5 CE, <LOQ = inferior to the limit of quantification. '+' positive, '-' negative, '?' presence of aspecific background, 'na' not available, '/' not examined. Number in brackets (n) refers to the day of slaughter after immune suppression.

2.7.5. Histological and immunohistochemistry procedures

Portions of the organs collected during necropsy were immediately fixed in 10% neutral buffered formalin and submitted to histological examination with the list of examined tissues reported in Table 2. Four-micron tissue sections were stained with hematoxylin-eosin (HE) and the microscopic findings were recorded for the typical EIA-associated lesions (OIE, 2013), hemosiderosis (HS) and lymphomonocytic infiltrates (LI) using the following score system, based on the degree of severity: '–' no lesions; '+' mild (few and scattered lesions); '++' moderate to diffuse (multifocal to disseminated lesions). As for the recording of clinical signs, the same pathologist registered all the histological alterations and had no access to the results of the other tests conducted on the same specimens.

In a preliminary trial, immunohistochemistry (IHC) was conducted principally on samples with evident histological lesions, using the following procedure: blocking of endogenous peroxidase with 3% H_2O_2 at room temperature for 30 min, antigen retrieving in citrate buffer solution (pH 6) in a water bath at 600 W in a microwave oven for 11 min, followed by an incubation with goat serum (Sigma-Aldrich, Inc., Missouri, US) at room temperature for 1 hour (h), washing in PBS followed by incubation with a primary monoclonal antibody (MAb) anti-p26 (Scicluna et al., 2013) for 1 h at room temperature. A positive reaction was detected using 3,3′-diaminobenzidine (EnVision™ FLEX, Dako, Santa Clara, CA, US) as chromogen with a 3-min development at room temperature and counterstained with haematoxylin. Samples examined and relative results are reported in Table 2 as presence (+) or absence (–) of a positive reaction.

3. Results

As the aim of the study was to evaluate the evolution of clinical, pathological parameters and viral loads of naturally EIA infected mules having equivocal/negative AGIDT reactions, the results of these animals (Group N – Mules 3, 5, 6, 7 and 8) were compared to those presenting a clearly positive AGIDT reactivity (Group P – Mules 1, 2, 4, 9 and 10).

3.1. Immune suppression

Dexamethasone administration was interrupted when the ratio between the DTH skinfold thickness, induced by PHA inoculation with that of the control reaction, was <1 (Kono et al., 1976 and Tumas et al., 1994) indicative of an immune suppressed state. The IS treatment was effective by day 8 in the eleven animals, with the exception of Mule 1, in which IS was obtained on day 10. Side effects due to steroid administration characterised by increased heart rate and laminitis were never observed.

3.2. Clinical, haematological and molecular results

The animals were clinically controlled and sampled on daily basis for 84 days, 56 days before and 28 days after the IS.

Samples that were examined to verify the presence of latent infections for EP and EHV 1 and 4 were confirmed as negative, except for those of Mule 6, that resulted positive for the former in a sample collected on day 12 Post-IS. Despite the spot presence of EHV 1 in this subject, it never presented clinical signs, rise in temperature or thrombocytopenia.

Fig. 1 reports the trends of rectal temperature, PLT and plasma-associated vRNA loads for the evaluation of the temporal association between these alterations relative to each study animal, including that of the negative control.

Hyperthermia, above 39 °C, considered the only clinical sign objectively correlated to the disease (Leroux et al., 2004), was observed between day 8 and 6 Pre-IS, in two mules reaching 40 °C and between day 9 and 17 Post-IS, in six subjects, with a maximum peak of 41.1 °C. In the febrile animals, hyperthermia lasted from one to five days maximum (Fig. 1). Clinical signs were generally absent for the whole observation period with the exception of Mule 3, that frequently presented a profound physical depression throughout the SP.

Following is a description of the alterations observed in the two groups.

Group N: Mule 3 had a constant presence of vRNA for the whole SP with a peak of $\log_{10}$ 5.10 copies/ml on day 17 Post-IS, concomitant with a febrile episode, and repeated PLT drops below the threshold level, especially in the initial period of the SP and in particular between day 50 Pre- and day 28 Post-IS. In addition to the aforementioned alterations of the sensorium, the subject presented frequent mild anaemia and jaundice with a progressive worsening of its general condition and for this was culled on day 28 Post-IS.

Mule 5 registered a viremic peak of $\log_{10}$ 6.88 copies/ml on day 17 Post-IS that was the highest value observed among all mules, accompanied by fever that was followed by thrombocytopenia lasting for 5 consecutive days, while mild anaemia was mostly present during the second part of the Post-IS period.

In Mules 6, 7 and 8, clinical signs were irrelevant and in the latter two, vRNA loads for the last two animals were detectable only occasionally, at lower levels to those observed in the other mules, between $\log_{10}$ 3.06 and 4.05 copies/ml, while Mule 6 was positive just on day 19 Post-IS, with a low vRNA value of $\log_{10}$ 1.97 copies/ml.

Group P: Mule 1 presented two viremic peaks, $\log_{10}$ 5.69 and 6.13 copies/ml, with the higher observed during the Pre-IS. On both occasions, the viral peaks were accompanied by fever and thrombocytopenia. Only on day 15 Post-IS did the animal present a moderate depression of the sensorium, in concomitance with the second viremic peak, accompanied by a PLT drop that lasted for seven consecutive days.

Mule 2 registered a viremic peak of $\log_{10}$ 5.4 copies/ml, the day following the end of IS, together with a transient low fever and thrombocytopenia, while anaemia was present at the end of the IS.

As for Mule 1, Mule 4 also presented a double vRNA peak, $\log_{10}$ 5.93 and 6.02 copies/ml, with the higher value recorded however during the Post-IS period and had a constant presence of vRNA during the whole SP. Repeated PLT drops in this subject were observed between day 22 Pre-IS and day 15 Post-IS. The same mule presented alterations of the sensorium, with mild depression, anaemia and oedema, subsequently to the second viremic peak.

Mule 9 presented detectable levels of vRNA constantly below $\log_{10}$ 2.96 copies/ml, between day 11 and 22 Post–IS, with just a PLT decline on day 14 Post-IS.

For Mule 10, two febrile episodes were observed; the first during the Pre-SP and the other on day 16 Post-IS, the latter was associated to thrombocytopenia, preceded by a vRNA peak with a value of $\log_{10}$ 3.91 copies/ml.

3.3. Total viral nucleic acid in tissues

The day on which the subjects were slaughtered, as also the results of the total viral nucleic acid/10^5 CE for the different tissues examined, are reported in Table 2. In the majority of the animals examined, the spleen had the highest viral load, followed by the spinal cord, liver, kidney and lymph nodes, with the exception of Mule 3 and 10, in which the highest total nucleic acid values were respectively found in the spinal cord and kidney. The remaining tissues were found positive with values less than 1.6 copies/10^5 CE or undetectable.

In Group N, total viral nucleic acid was also detected in the brain and adrenal glands of two different subjects, Mule 3 and 5, as well as, in the lymph nodes of all animals except for Mule 6. In addition, in this group, the positive animals had overall, higher values than those obtained for the other group, especially for the spleen tissue of Mule 5, with 487 copies/10^5CE. For Group P, the maximum values were detected in the spleen of Mule 10, with 60.83 copies/10^5 CE.

Results of vRNA in nasal swabs were as following: in Mules 1, 2, 4, 5, 6 and 10, detection was sporadic (one to three samples) with vRNA loads close to the detection limit, ranging from 0.002 and 10.74 copies/ml which were recorded between day 1 and 21 Post-IS and were not simultaneous to the plasma vRNA peaks.

3.4. Verification of the presence of PCR inhibiting activity

Amplification of the endogenous gene coding for Beta-actin was successful in all the samples that were examined by the different PCRs described above.

3.5. Bacteriological examinations

No bacterial examinations were carried out on the nasals swabs as there were no signs referable to respiratory diseases during the entire SP.

3.6. Gross, histological and immunohistochemical results

No relevant gross lesions were found at necropsy. The principal microscopic lesions observed are reported in Table 2. Moderate HS in the cytoplasm of macrophages was evident in the spleen of all animals, except for Mule 2. With a lesser degree of severity and at a lower rate, the same alteration was observed in the Kupffer cells and in the lymph nodes macrophages. In the liver of two subjects, lymphomonocyte infiltrates (LI) were present in the periportal duct areas. In the kidney of all subjects examined, LI were present with evidence of mild to moderate membranous proliferative glomerulonephritis in four subjects.

LI was almost constantly present in the lungs examined and occasionally associated with the accumulation of lymphocytes at the peribronchiolar area together with a mild thickening of the alveolar septa. Similar inflammatory lesions of modest entity were also evident in the cardiac interstitium of seven mules. LI were also present in the brain of three animals, Mule 1, 3 and 6, prevalently organised as lymphocytic perivascular cuffings in the white matter (lymphomonocytic leucoencephalitis): in Mule 3, LI were more diffuse with respect to the other two subjects and were also associated with a severe multifocal lymphocytic meningitis. Other lesions observed were the presence of scattered foci of lymphocytic nodules in the adrenal gland of one subject of the five examined and a multifocal or diffuse degeneration of the hepatocytes of Mule 2 and 8.

Preliminary IHC, in some of the tissues examined belonging to three subjects of Group P and four subjects of Group N, detected p26 antigen in the cytoplasm of a few scattered macrophages in the inflammatory lesions of the kidney, liver, lung, heart and adrenal gland (Fig. 2).

The negative control animal did demonstrate any alterations for the clinical, haematological and histological parameters and was constantly virologically and serologically negative for EIAV.

4. Discussion

The current study reports the first description relative to the evolution of the clinical, quantitative virological and pathological findings in ten asymptomatic, naturally infected mules, presenting different AGIDT serological reactivity patterns that were subjected to pharmacological immune suppression for the reactivation of the EIA infection. On enrolment, none of the mules were presenting clinical signs referable to EIA, nor were they in the viremic phase of the infection as all of them were reacting negatively for the presence of plasma vRNA. The animals were however confirmed as infected by EIAV, as proviral cDNA was detected in their buffy coat, using a conventional PCR as described by Cappelli et al. (2011) and, as mentioned further on, products of this PCR were used for a preliminary sequencing of the strains involved. This condition is ascribable to an inapparent state of infection. In further support of this, EIA serological diagnosis had been carried out at least six months prior to the beginning of the SP and even then, the study animals were in the inapparent phase of the disease. This status is similar to that described by Hammond et al. (2000) for EIA persistently infected horses during the asymptomatic phase, in the absence of stress related conditions (Harrold et al., 2000).

As in other studies conducted for the investigation of EIAV replication, through the quantitation of the vRNA loads in plasma from which the cellular part was removed, the PCR could have also amplified proviral DNA that could have been present in the sample. In our case we did not include treatment with DNAase, as also omitted by other authors (Quinlivan et al., 2007), as in trails we conducted there was a decrease in the amount of the vRNA amplified. Even in the absence of DNAase treatment, the amount of proviral DNA is minimal because, as stated in the introduction, EIAV replication occurs only in differentiated tissue macrophages. In addition, also as reported by Harrold et al. (2000), during the different disease states, peripheral blood mononuclear cells were found to contain less than 1% of the cellular viral burden.

During the present study, active viral replication began in five mules belonging to both AGIDT serological groups (Fig. 1) during the Pre-IS period and coincided to when the subjects were put together. Such an event presumably represented a stress due to the new management these were experiencing. All the animals were subsequently subjected to an experimentally controlled immune suppression, using the same protocol employed in horses (Kono

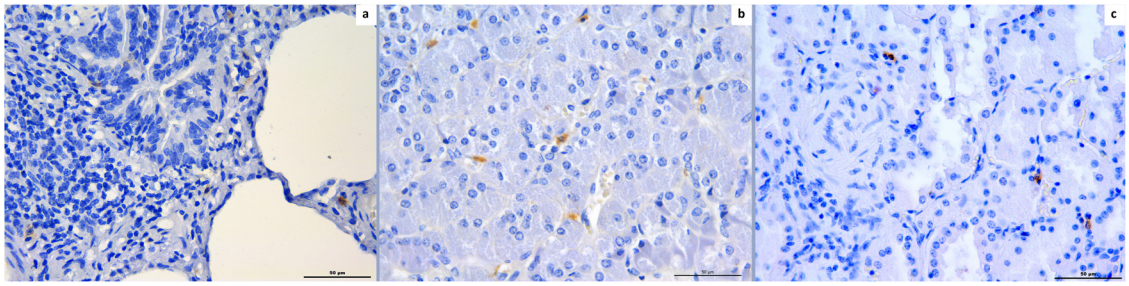

Fig. 2. IHC positive reaction: (a) Lung (Mule 5), (b) Adrenal gland and (c) Kidney (Mule 10). Scattered cytoplasmic immunoreactivity in mononuclear cells morphologically resembling macrophages, randomly distributed in (a) the alveolar septa, in (b) the pars intermedia of the adrenal gland and in (c) the renal interstitium. In the lung (a) and in the kidney (c) the immunoreactivity is detectable within the lymphoplasmacytic infiltrates. Anti-p26 Mab, Hematoxylin counterstain, 40×.

et al., 1976; Tumas et al., 1994; Craigo et al., 2007), that provoked vRNA peaking in all of them, even if in one subject, virus replication was detected just on one day. Administration of dexamethasone in these animals proved to be effective in inducing immune suppression as verified by their delayed hypersensitivity reaction. This condition caused the recrudescence of EIA simultaneously to IS, with reactivation or increase of EIAV replication, that reached levels higher than those observed in the Pre-IS period, accompanied by fever and PLT decline in nine of ten study subjects mules. Differently from what was observed in similar experiments conducted in horses (Craigo et al., 2007; Tumas et al., 1994; Kono et al., 1976), in our study, viral peaking was exclusively accompanied by mild signs that only a careful daily clinical examination was capable of detecting. With the exception of Mule 3, which was continually depressed, neither the high temperature, that was however transient, nor the thrombocytopenia recorded in the other nine mules profoundly altered their general state. If a pathogenic threshold was to be determined in the present study, as reported for the horse, this is between $\log_{10}$ 4.20 and 6.88 copies/ml of plasma vRNA, which is inferior to that reported by Cook et al. (2003). Such lower levels could be due to the use of a different PCR method with a lower recovery rate to that reported by the same authors (Cook et al., 2002).

Although the clinical findings observed throughout the SP corroborated the inapparent phase of infection, they did not correlate with the dynamics of EIAV replication, typical of its reactivation.

On the whole, our mules presented a clinical behaviour similar to that described in donkeys which have plasma vRNA burdens that are 1000-fold less than that of the horse (Cook et al., 2001), but with vRNA peaks that can be described as closer to those reported in acute forms or reactivation of EIA in the latter species.

vRNA replication behaviour observed in the present study is another important feature in the evolution of EIA infection in these animals, as there were important fluctuations in the plasma viral levels, with some animals having periods of vRNA negativity, during which testing of the plasma by molecular methods could have falsely led in classifying them as negative.

When comparing the vRNA replication levels throughout the study period in the two groups, even if the proportion of positive samples were greater for Group P, the interval of the plasma vRNA values observed was however similar; within a range of $\log_{10}$ 2.0–6.8 copies/ml for Group N and $\log_{10}$ 3.0–6.1 copies/ml for Group P, with the highest titre observed in the former. On the basis of these observations, it would seem that viral replication is independent of the serological reactivity, at least for p26, which is the antigen used in the AGIDT, as EIAV replication was indifferently observed within the animals belonging to the different groups. This result is in partial contrast with the assumption that the EIA immune status depends on its antigenic stimulation (Cook et al., 2003). In our case, there were mules, Mule 3, 5 and 8, that even if experiencing a continuous EIAV replication, showed an AGIDT antibody response continually low and not attributable to what could be an initial phase of the infection (Table 1).

For this reason, mules having an equivocal serological response and with fluctuating vRNA plasma loads could pass undetected when using tests of low diagnostic sensitivity, as is the case of the AGIDT (Issel et al., 2013) and also in some cases the PCR when virus levels are below the detection limit and/or due to the intrinsic genetic variability of EIAV.

Relative to the quantitation of the total viral nucleic acid loads, we adopted a method that even if different from that described by other authors in horses (Harrold et al., 2000) and in mules (Spyrou et al., 2003), gave overall results generally in agreement with those reported by these authors, with the exception of the nervous tissue, for which discordant results for the horse exist (Harrold et al., 2000; Kim and Casey, 1992; Kono et al., 1971). Even in the mule, as also reported by Quinlivan et al. (2007) and Harrold et al. (2000) for the horse, the highest viral nucleic acid loads were constantly detected in the spleen tissue of all subjects examined, with the exception of Mule 3 and 10.

Of note is that the greatest tissue viral nucleic acid loads were observed in two animals of Group N, Mule 3 and 5, that are in agreement with their highest vRNA plasma levels. Of particular interest is whether the viral amounts detected in the brain and spinal cord tissue of Mule 3, as well as the total viral loads and histological lesions at this level, are correlated with the severe depression of the sensorium the subject presented throughout the study and was for this the first to be slaughtered.

In relation to the persistence of EIAV in tissues, moderate to high levels of viral nucleic acids were still detectable even in Mule 2 and 4, slaughtered between 30 and 60 days from when they had experienced their highest vRNA peaks. A further confirmation of this is that animals in which viral nucleic acid was absent corresponded to those with relatively lower levels and proportion of positive samples for plasma vRNA, even if this could be due to a low sensitivity and/or specificity of the PCR method employed for the strains involved that should be further analysed. In fact for Mule 6, the animal with the lowest virological/serological reactivity, these results could be attributed to a particular strain of EIAV or to the capability of the animal of efficiently controlling the infection. This hypothesis may be supported by the presence of the p26 antigen observed with IHC in the lung, adrenal gland and liver of the same animal.

A preliminary study that we conducted on the sequencing of the gag gene of the EIAV strains of the study animals, using the method reported by Cappelli et al. (2011), Mule 6 had a sequence identity of 81% with EIAV Rom-4 (GU060662.1) while the other strains had a sequence identity that varied from 77 to 96% to previously identified European EIAV strains. Further phylogeny studies are being carried out on the EIAV strains involved in this study and will be discussed in another article.

Persistence of EIAV for long intervals at tissues levels, as well as in the plasma, implies that notwithstanding the inapparent state of infection and a limited AGIDT reactivity, animals may act as efficient reservoirs in which viral replication reaching high levels can be retriggered under particular conditions such as those described in this paper.

The microscopic lesions observed in the various tissues are similar to those reported in the chronic forms of EIA in ponies (Oaks et al., 2004) and mules (Spyrou et al., 2003) as also the IHC positivity to what was described by Bolfa et al. (2013) for the horse, using immunofluorescence. In relation to this, lesions and IHC reactivity described in the lung require further investigations to verify the possible viral release through the respiratory tract, a transmission route not previously considered for EIA. Even if our findings do not support this assumption, as low viral loads were detected in the nasals swabs of our animals, this is in contrast to what was described by Quinlivan et al. (2007) who reported higher amounts of RNA copies/ml in nasal swab material in symptomatic horses. Detection of different levels of vRNA in this sample type could be due to the species involved or time since infection, which in the case of the Irish outbreak, was very recent, or also, as previously mentioned, due to the sensitivity of the PCR method, employed.

Further to what was reported by Spyrou et al., 2003; this paper describes the presence of inflammatory lesions in the myocardium and brain of some of the animals examined. Pathological alterations in the brain tissue defined as granulomatous ependymitis (McIlwraith and Kitchen, 1978) or as periventricular lymphohistiocytic leukoencephalitis (Oaks et al., 2004) were occasionally described for horses and ponies but not considered as relevant in an EIA infection. Preliminary trials using IHC confirmed that the histological lesions

were EIAV-associated except for the alterations observed in the nervous tissue that lack confirmation due to the presence of an aspecific background. Relative to the absence or low amounts of viral nucleic acid in tissues that presented histological lesions and/or IHC positivity, this could be attributed to the degradation that the former could undergo during sample storage or to the sensitivity of the PCR method, in terms of detection limit and the possible genetic variability of the PCR target. On the other hand, the IHC method has as target, an antigen that is less degradable than genetic material and is also highly conserved in EIAV, as only one serotype is currently known for this virus.

In this study, it was not possible to correlate parameters such as age and breed with the evolution of the EIA infection as the set of animals within the groups were heterogeneous. Relative to the sex of the animals, in the mule population, it is the females that are kept as working animals as they are more docile and therefore our study group is representative of the field population structure. In addition, the identification number was arbitrarily assigned according to the succession of the recruitment time; therefore the fact that Mules 1–5 had a more intensive evolution of EIA is casual. As mentioned earlier, even the viral strains infecting these subjects were to some extent genetically different. The evolution of EIA in our study is on individual basis and is a result of the multiple host and viral factors that are in this context difficult to identify but which however does not correlate with the type of AGIDT reactivity.

According to the current European Union Directive (2009/156) on the movement of equidae between member states it is sufficient to declare freedom of disease just on the absence of clinical signs. In the light of the inapparent phase of EIA infection described in horses and mules, such an affirmation does not fully guarantee the true health status of the animal, which also might not be accurately defined with the sole use of AGIDT. For this, the perception that would rise from the use of a passive surveillance is that in the absence of clinical signs, or a negative AGIDT reaction, an EIA infected equid represents a low transmission risk.

The increasing knowledge on the evolution of EIA, even in mules, as also the data generated from the surveillance conducted in Italy over a period of six years (Scicluna et al., 2013) provided the basis for the set up of an active risk based serological surveillance, using the ELISA as a screening test.

Competing interests

The authors declare that they have no competing interests.

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