SINTESI (SUMMARY) (da redigere in lingua inglese secondo le indicazioni riportate per la sintesi in italiano, senza la ripetizione di grafici, tabelle e bibliografia)

Title:
Study of the role of the innate immunity of the horse in the control of equine infectious anemia infection

[Text]
The Equine Infectious Anemia virus (EIAV) belongs to the *Lentivirus* genus, *Retroviridae* family. Within the same genus there are, in addition to the human immunodeficiency virus (HIV), other important pathogens of veterinary interest such as the Bovine Immunodeficiency virus (BIV), the Feline Immunodeficiency virus (FIV) and the Lentivirus complex of Small Ruminants (SRLV).
Lentiviruses are species-specific and innate immunity would be at the basis of the species barrier and the mechanisms of transgression and blocking. As described for small ruminant lentiviruses, this could also occur against homologous lentiviruses. The activity of innate immunity is exercised early with respect to the response of the adaptive system, as it is able to identify molecular patterns specifically associated with pathogens through different molecules with the function of recognition receptors of the same. Following recognition, the transduction signals of immune cells (macrophages and/or dendritic cells) induce a series of events capable of counteracting the infection. The proven recognition of the effective inhibition of the viral replication cycle is the basis of the increased interest in the intrinsic factors of innate immunity. The scientific literature reports studies on the role of the innate restriction system in the control of lentiviruses in small ruminants and also HIV, however, there is no similar research in the equine model regarding EIAV. Furthermore, for the latter, phylogenetic studies limited to some portions of the viral nucleic acid are available in the literature, while there are few sequences of the entire genome capable of providing useful information also for diagnostic purposes.
The general objective of the project is the direct / indirect study of the presence in the horse of the genes responsible for the innate restriction system and the *in vitro* evaluation of its mechanisms of action against EIAV.

[Objectives]
The objectives of the project were the following: identification of the equine population for the purposes of the study; isolation of viral strains circulating nationally; molecular characterization of the field strains; isolation and characterization of macrophages starting from different biological matrices; evaluation of viral production and expression of polarization markers.

[Brief summary of the methodology]
Population selection to be sampled
Investigations for interfering viruses such as EHV2 and other equine herpesviruses (EHV1, EHV4, EHV5)
Isolation of macrophages from the spleen and peripheral blood
Viral isolations and amplification of macrophage and serum-plasma strains
Characterisation of the viral genome in the absence of culture isolation.
Verification of RT activity from the supernatant of macrophage cultures and from the serum / plasma of infected subjects
Data analysis for the molecular characterization of the jambs with particular attention to

epidemiological aspects
Updating of the phylogenetic and evolutionary relationships of the circulating strains with respect to the deposited references
Characterisation of polarization markers by real time PCR
Confirmation of natural immunity and restriction factor markers with quantitative RT-PCR
Characterization of expression patterns following infection with reference and / or field strains
Quantification of viral production levels in polarized macrophages
Training of UUOO personnel on the aspects of their own competence

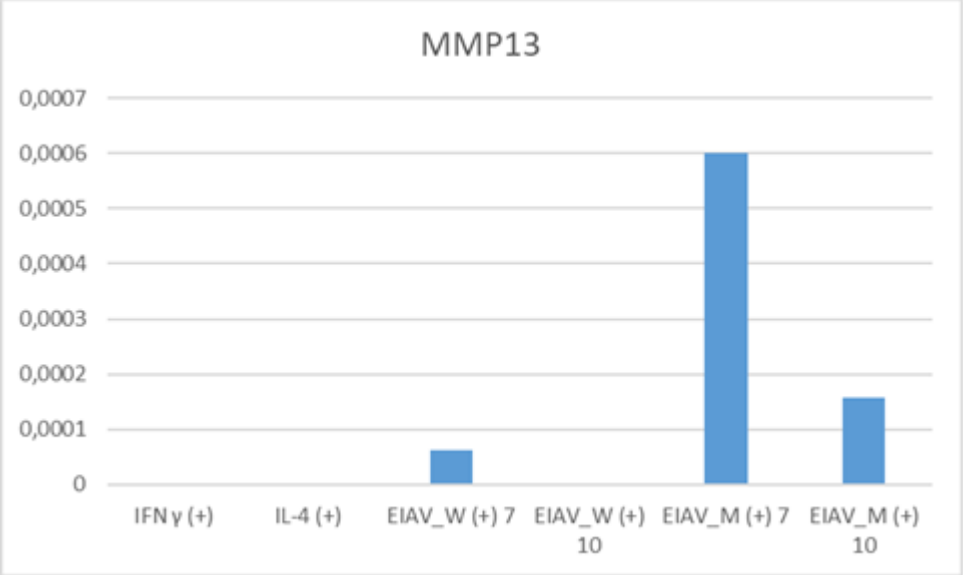
[Results]
The results of the research activity were as follows:
• development of standardized protocols for the isolation and culture of a virus not sufficiently studied until now due to the difficulties encountered using common in vitro virological methods;
• isolation of field strains in macrophages isolated from the spleen and peripheral blood of horses seropositive for EIAV
• verification of the differentiation capacity of macrophages into subpopulations following stimulation with cytokines and consequent expression of the genes responsible for the restriction mechanisms in healthy and naturally infected animals;
• characterisation of viral strains isolated by Sanger sequencing and NGS for the purposes of phylogenetic studies and for the development of molecular methods with high diagnostic sensitivity.
• characterisation of macrophage line polarization patterns in uninfected horses by stimulation with IFN-γ (M1) and IL-4 (M2)
• all samples tested (biological material and macrophage culture supernatants) were negative in PCR for equine herpesvirus.

[Brief discussion and conclusion]
As part of this research project, isolation and *in vitro* cultivation protocols of equine macrophages were developed, which allowed to obtain cells from which it was possible to proceed with the amplification and sequencing of almost the entire genome of the EIAV, using the NGS.
The biological matrix found to be most effective for the isolation and cultivation of macrophages for the replication of the EIAV is represented by whole blood with anticoagulant, with better results compared to spleen sampling.
Preliminarily, from the sequencing obtained with the different methods it was possible with the use of the Nested PCR *LTR-tat* protocol to correlate the Italian EIAV strains with those identified in Mongolia and Austria, denoting a wide viral circulation.
Instead, the sequence of the genome, obtained with the NGS technique, and in particular for the gag gene, indicates that the EIAV detected is relatively divergent from the strains present in the available databases, in fact, the greater similarity based on gag is to be referred to strains French characterized in 2009 (84.2% similar).
The significant divergence found at the TM protein level between the Frosinone isolate and the Wyoming reference strain deserves further study. In fact, this sequence is often incorporated as a diagnostic antigen in some commercial kits and may not be able to detect antibodies from infected subjects with such divergent strains. It will therefore be useful to generate synthetic peptides corresponding to the strains circulating in Italy for a verification of the diagnostic performance, especially in equine populations residing in areas affected by recent outbreaks.

For the activity related to the obtaining of macrophage cultures of uninfected horses for the purpose of evaluating the polarization in the different subpopulations by stimulation with IFN-γ (M1) and IL4 (M2) the execution protocols (isolation of macrophages, stimulation with equine cytokines, infection with EIA virus) developed. They revealed that peripheral blood monocytes always differentiate into macrophages, often giving rise to mixed populations of M1-like and M2-like. The addition of specific cytokines or a viral antigen determines a more specific differentiation.
In our experiments, equine cytokines of the trade Interferon-gamma (IFN-γ) and equine recombinant Interleukin 4 (IL4) were used: stimulation with cytokine IFN-γ polarized the macrophages towards the M1 isoform (fried egg, with more or less irregular shape); stimulation with cytokine IL4 polarized the macrophages towards the M2 isoform (emission of long pseudopodia, similar to fibroblastic cells).
Viral infection (EIAV Wyoming strain and Miami strain) caused the morphological transformation of macrophages into M1.
For the purpose of studying the polarization of equine macrophages following stimulation with cytokines and viruses, it was possible to verify that the detection system with the chosen primers and SybrGreen PCR works. While the interpretation of the results is ongoing, we will continue to identify new primer pairs for new genes in order to broaden the expression study and lay the foundations for the subsequent development of a method in NGS.
Unfortunately, two years of the COVID-19 pandemic have significantly slowed down the execution of the activities envisaged by the project as the IZSLT Virology laboratory (UO 1) is one of the laboratories in charge of carrying out the molecular diagnostics of SARS-CoV-2. For this reason, it was not possible to complete some activities foreseen by the project.
A more in-depth study of the expression genes of the eMDM polarization markers, both cytokine-induced and virus-induced, will help us to understand if these markers are the same in horses as those found in other animal species (pigs, sheep, goats, humans) with similar modes of activation of innate immunity against lentivirus. The quantification of the virus in the infected wells will help us understand the pro-inflammatory (restrictive) or anti-inflammatory (permissive) activity of the eMDMs polarized in the two isoforms depending on the cytokine used, against the infectious anemia virus of the horse. All this to understand the action performed by the horse's innate immunity following infection with EIAV.

[Most significant graphics]

Expression of proinflammatory relative to markers (MMP13 and IL6) in seronegative horse macrophages after stimulation with cytokines and with EIAV strains Wyoming (W) or Miami (M) at 7 and 10 days


MMP13
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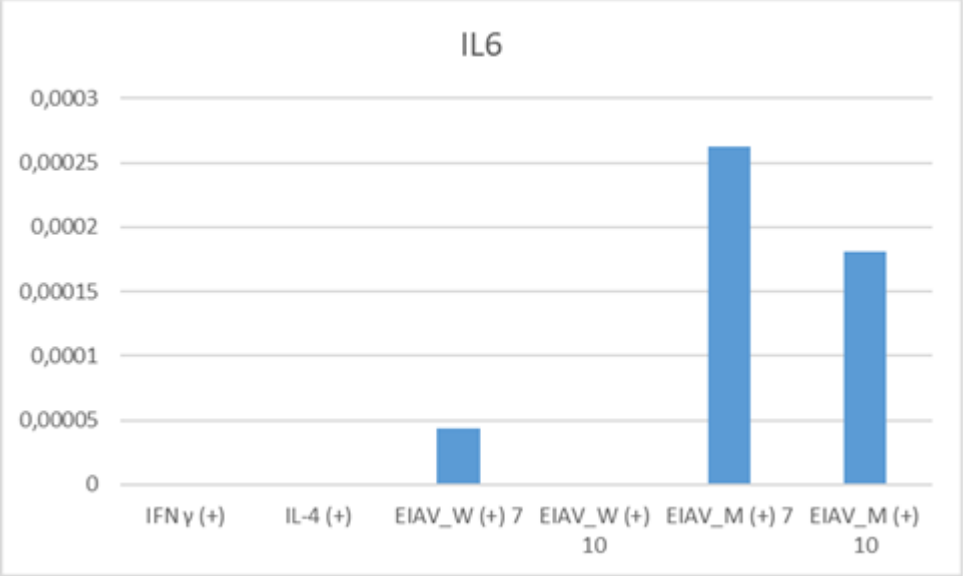
0,0006
0,0005
0,0004
0,0003
0,0002
0,0001
0
IFN γ (+)
IL-4 (+)
EIAV_W (+) 7
EIAV_W (+) 10
EIAV_M (+) 7
EIAV_M (+) 10


IL6
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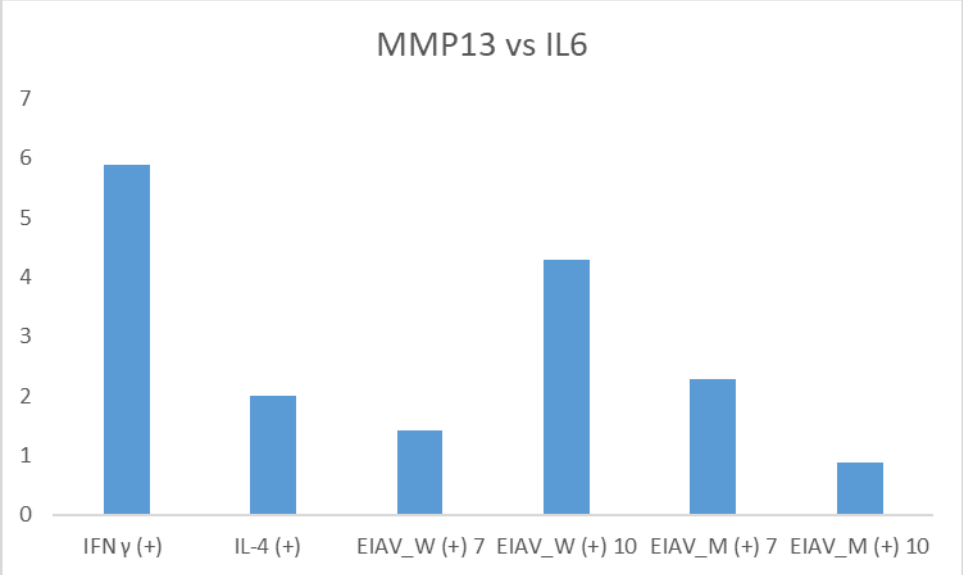
0,00025
0,0002
0,00015
0,0001
0,00005
0
IFN γ (+)
IL-4 (+)
EIAV_W (+) 7
EIAV_W (+) 10
EIAV_M (+) 7
EIAV_M (+) 10



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Horse, innate immunity, equine infectious anemia virus, cytokines, PCR, phylogeny, polarization

[Indications to allow the citation of the report (authors, reference e-mail, year, title, etc.)]

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