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Evaluation of siRNA antiviral activity against different Influenza A virus biotypes

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In recent years, concerns related to human health caused by Influenza A virus (e.g. pandemic Influenza virus H1N1) have highlighted the relevance of the animal host. In fact, it is well known it could act as *reservoir* in the transmission of this pathogen to humans. In particular, swine has a key role in the potential recombination between different biotypes of influenza virus.

Moreover, the appearance of new more virulent biotypes of Influenza virus, able to make ineffective the standard vaccination protocols, has driven studies towards new drugs/molecules with antiviral activity. In this field, the phenomenon called RNA interference (RNAi) covers a particular interest. Actually, many recent publications have demonstrated that *short interfering RNAs (siRNAs)* are able to act as antiviral molecules, inhibiting the translation of particular ribonucleotide sequences and, therefore, the synthesis of proteins essential for viral replication.

In the present study, our purpose was to evaluate if the constitutive expression of single siRNA sequences in *in vitro* cell cultures can cause the inhibition of replication of different influenza A viruses. Therefore, we have selected conserved sequences on the nucleoprotein (NP) region as targets for siRNAs potentially effective against different biotypes.

In order to determine the most effective siRNA sequences, a preliminary screening by luciferase assay was developed. Then, MDCK (*Madin Darby Canine Kidney*) cells were stably transfected with DNA vectors expressing the best siRNAs. Subsequently, clones underwent to infection with the following swine influenza A virus (SIV) biotypes: *A/SW/1521/98, H1N2; A/SW/1523/98, H3N2* and *A/SW/1513/1/98, H1N1*. Moreover, we have used the avian Influenza A virus biotype *A/TK/Italy/2676/2000, H7N1*. Two siRNA sequences have demonstrated high ability to inhibit viral replication *in vitro*. In fact, none of the infected cell samples has shown a cytopathic effect. Parallel, *Real-Time RT-PCR* has confirmed same results. This technique allowed us to obtain a strong down-regulation of NP *mRNA* in samples expressing specific siRNA.

Therefore, this study has demonstrated the siRNA efficacy in the inhibition of *in vitro* replication of different viral biotypes of swine and avian Influenza A, suggesting other different features need to be analyzed. Firstly, we have to test siRNA efficacy against human and other avian Influenza biotypes. Secondly, we have to evaluate the real ability to inhibit viral replication through *in vivo* tests. This is of relevant importance because the use of siRNAs could represent an innovative and operative therapeutic approach in human and veterinary medicine.

Vaccine development for Bluetongue and African HorseSickness using reverse genetics: similarities, differences and limitations

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Bluetongue (BT) and African horseSickness (AHS) are notifiable diseases of respectively ruminants and equines, caused by different virus species within the genus *Orbivirus* of the *Reoviridae* family. The course of disease is fast and mortality of AHS in domestic horses can be extremely high (>90%). These orbiviruses are transmitted by bites of specific species of Culicoides midges. Spread of BTV8 in Europe by Culicoides species endemic in a moderate climate has initiated re-evaluation of the emergence of orbiviruses in Europe. Evidently, introduction of BTV or AHSV will have a huge economic and socio-emotional impact on the entire community, whereas control of vector-borne diseases is hardly possible without vaccination. Currently, there are temporarily produced inactivated BT vaccines available for certain serotypes, like in Europe for serotype 8 and a few others. For AHS, there is no registered vaccine available outside Africa. Conventionally developed live-attenuated vaccines are considered unsafe due to several reasons; under-attenuation, restored virulence by reassortment or by 'reverse to virulence', and lack of DIVA characteristics.

Reverse genetics for BTV enabled *de novo* generation of genetically modified BTV resulting in a next generation vaccine candidate for serotype 8, named Disabled Infectious Single Animal (DISA) vaccine 8 (Feenstra et al., 2014). BT DISA vaccine is based on the vaccine-related BTV6/net08 in which immunogenic NS3/NS3a is deleted. This resulted in complete avirulence, local replication and a strongly reduced viremia of vaccine virus, and DIVA characteristics. Exchange of immunodominant VP2 resulted in serotype specific protection (Feenstra et al., 2014). Exploring of this BT DISA platform for other serotypes will be updated.

Accordingly, reverse genetics based on vaccine virus AHSV4LP (Erasmus 1973) was used as a platform for AHS DISA vaccines. We abolished NS3/NS3a expression and exchanged VP2, resulting in a set of AHS DISA vaccines for all nine serotypes. These are not tested in horses yet, but we assume, in line with BT DISA vaccine 8, that these are safe, efficacious, and will enable DIVA based on immunogenic NS3.

So, BT and AHS DISA/DIVA vaccine candidates are both based on a safe vaccine virus and are negative for NS3/NS3a expression. Since only the serotype determining VP2 protein was exchanged, reassortment between DISA vaccines of different serotypes cannot result in new variants. Further, DISA vaccines for different serotypes will be very similar, and thus can be randomly and safely administered or combined in cocktails.

Studies on DIVA characteristics of DISA vaccines are in progress, and a competition ELISA based on NS3 is developed for BTV. Similarities, differences, and limitations of DISA vaccines for Bluetongue and African HorseSickness will be discussed.

The comparison of the efficacy of swine FMD vaccine emulsified with oil adjuvant of ISA 201 VG or ISA 206 VG

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The Seppic Company developed a new adjuvant Montanide ISA201 VG, the upgraded version of Montanide ISA 206 VG, which keep the advantage and added some chemical components on the basis of ISA 206 to improve the cellular responses. The aim of the study is to compare the efficacy of swine FMD (foot-and-mouth) vaccine emulsified with oil adjuvant of ISA 201 or ISA 206 respectively.The pigs were vaccinated with FMD vaccine emulsified with inactive FMD type O antigen and adjuvant ISA 201 or ISA 206 respectively, according to 2.0ml(1/1 dose),0.67ml(1/3 dose),0.22ml(1/9 dose) to calculate their PD50. The sera were collected from the vaccination of the day 0, 3, 7, 14, 21, 28 and the ELISA FMD type O antibody were detected. Furthermore, the PD50 were calculated after the pigs were challenged with virulent FMDV type O on 28 days post vaccination. The ELISA antibody titers of 201vaccine were significantly higher than that of 206(except the third time). The fifty percent of protection dose (PD50) of 201 vaccine (PD50=15.59) was higher than that of 206 vaccine (PD50=10.05). The above data showed that the efficacy of the FMD vaccine emulsified with ISA 201 was better than which with ISA 206.

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Modification of FMDV anti-host defense mechanism

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Foot-and-mouth disease virus (FMDV) is the etiologic agent of FMD, an infectious and sometimes fatal viral disease that affects cloven-hoofed animals. The FMDV genome encodes a large polyprotein, the first component of which is the Leader protein. Unusually, within the picornavirus family, the FMDV Leader protein (L^{pro}) is a protease. This protease induces a very rapid inhibition of host cell cap-dependent protein synthesis within infected cells. This results from cleavage of the cellular translation initiation factor eIF4G. Translation of the viral RNA is unaffected since it is dependent on an IRES that directs cap-independent translation initiation. L^{pro} also releases itself from the virus capsid precursor (at the L/P1 junction). By site-directed mutagenesis of the L^{pro} coding sequence, amino acids that are involved in inducing cleavage of eIF4G and hence blocking cap-dependent translation are being identified. Among these, we aim to identify mutants that maintain the L/P1 junction cleavage activity. This study may allow design of mutant viruses that are deficient in blocking host cell responses to infection (e.g. interferon induction) and assist in the rational design of antiviral agents targeting this process.

Sequence adaptation during growth of modified classical swine fever viruses in cell culture

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Classical swine fever (CSF) is an economically important, highly contagious porcine disease caused by classical swine fever virus (CSFV). The 5ˈ-untranslated region (5ˈUTR) of CSFV contains an internal ribosomal entry site (IRES) directing the cap-independent initiation of protein synthesis. However, rather little is known about the effects of mutations within the 5ˈUTR on virus viability and growth. IRES mutants containing modified stem2 structures within the pseudoknot (domain IIIf) have been described previously (Friis et al., 2012). Viruses containing these mutations were examined for sequence adaptations during growth within infected cells. The mutant viruses were serially passaged in porcine PK15 cells and the 5'UTR of the CSFV RNA was amplified by RT-PCR from selected passages (P-5, P-10, P-20) and sequenced. The 5'UTR sequence of the rescued parental virus (vPader10) remained stable but some adaptations occurred within the pseudoknot region of the mutant viruses, which appeared to stabilise the mutant IRES structure. The identified adaptations within the IRES region were confirmed by next generation sequencing (NGS) analysis of the complete viral genomes. In addition to the stabilised IRES structures, a single nt sequence change (G to A at nt 11,864) within the coding region for the NS5B protein (the RNA polymerase) was highly represented after 5 passages (>90%) in the virus population and by P-10 was present in essentially 100% of the viral RNA molecules. This demonstrates a strong selection pressure for this change which returns the cDNA clone derived vPader10 sequence to the consensus sequence of the Paderborn virus (#AY072924).

Friis MB, Rasmussen TB & Belsham GJ. (2012). Modulation of translation initiation efficiency in classical swine fever virus. J. Virol 86, 8681-8692

Development of a harmonized real-time RT-PCR for the detection of normalized gene expression profiles of seven porcine cytokine

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Dysregulation of cytokine responses plays a major role in the pathogenesis of severe and life-threatening infectious diseases like septicemia or viral hemorrhagic fevers. In pigs, diseases like African and classical swine fever are known to show exaggerated cytokine releases. To study these responses and their impact on disease severity and outcome in detail, reliable, highly specific and sensitive methods are needed. For this reason, a harmonized TaqMan-based real-time RT-PCR for the quantitative detection of normalized gene expression profiles of seven porcine cytokines was designed and validated within the presented study. Cytokines were chosen to represent different immunological pathways and targets known to be involved in the pathogenesis of the above mentioned porcine diseases, namely interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, tumor necrosis factor (TNF)-α and interferon (IFN)-α. Beta-Actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as reference genes for normalization. For absolute quantification a synthetic standard plasmid was constructed. The standard as well as positive RNAs from samples, and additionally more than 400 clinical samples, which were collected from animal trials, were included in the validation process to assess analytical sensitivity and applicability under routine conditions. The resulting assay allows the reliable assessment of gene expression profiles and provides a broad applicability to any kind of immunological research in swine.

Control of a reassortant H1N1pdm SIV infection in a swine farm in Italy

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Swine influenza viruses (SIVs), are viruses belonging to the family *Orthomixoviridae,* genus *Influenzavirus A*, characterized by a segmented genome of 8 single-stranded RNA molecules. SIVs are classified on basis surface glycoproteins into 18 hemagglutinin and 10 neuraminidase. In the Italian pig population 3 SIV subtypes, antigenically related to human influenza, are mostly found: H1N1, H1N2 and H3N2. Besides, H1N1pdm2009 circulation has been recently described. From 2010 to 2012, an outbreak of respiratory disease with severe morbidity (75%) and a high case fatality rate (25%) was reported in 60-day-old pigs in a breeding farm in Northern Italy. Animals were kept under a continuous-flow cycle with weekly batches, without all in/all out management. Treatments with anti-inflammatory and antibacterial drugs had limited or no effect. Virological analyses conducted in the same farm (Chiapponi *et al*., 2013) highlighted the presence of an H1N1virus strain evolved from the pandemic H1N1 virus and an avian-like H1N1 pig strain through natural reassortment. To control the outbreak, in July 2013, the inactivated vaccine FluSure Pandemic (against subtype pH1N1, A/California/04/2009 isolate) was introduced under derogation in Italy. Before vaccination, further analyses were performed to confirm the circulation of H1N1pdm. Nasal and saliva swabs collected from 75 piglets with fever and respiratory signs, were analyzed for SIV-RNA, using Real-Time RT-PCRs targeting two different genes, matrix and specific H1N1pdm haemagglutinin (Hoffmann *et al*., 2010). All positives were further subtyped using RT-PCR (Chiapponi *et al*.,2012). Five pigs out 75 (6.6%) tested positive for H1pdm in at least one sample type by Real-Time RT-PCR. In October 2013 a vaccination plan started: all sows were vaccinated at 6 and 3 weeks before farrowing and all gilts at 6 and 3 weeks before fecundation. All vaccinated animals were treated again with a single booster 3 weeks before the following farrow. In order to evaluate the vaccination efficacy in sows, antibody titers against H1N1pdm were quantified in a sample of their piglets (2 randomly selected groups of 20 piglets each). Blood samples were collected once every ten days between 1 and 3 months of age. At one month, 95% of piglets in group 1 and 70% in group 2 showed protecting antibody titers. A progressive reduction of maternal immunity was detected. However, at two months of age, 70% of piglets in group 1 and 44% in group 2 still had detectable antibodies. From December 2013 to April 2014, nasal and saliva swabs were collected from symptomatic and apparently healthy piglets born from vaccinated sows. Five sampling rounds were performed aimed at investigating H1N1pdm circulation after vaccination. All samples were analyzed as described above. Only 1/129 animal (collected in December 2013) was tested positive for H1N1pdm in both nasal and saliva swab. Eight months after vaccination, a significant decrease of mortality rate (5%) was observed in piglets. Besides vaccination, farm management was improved. Piglets born from vaccinated sows were weaned at 28 days, prolonging the turnover of animals in the barns. This, coupled with sow vaccination, allowed for the controlof virus circulation in the farm.

Immunization with DNA constructs encoding native and chimeric proteins of Rift Valley Fever virus

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Rift Valley fever (RVF) is a zooanthroponotic viral infection currently circulating in many countries of Africa and Middle East. Wide range of both wild and domestic animals are susceptible to RVF virus including cattle, sheep, camels, goats, and humans. Nowadays, design of genetically engineered vaccines is prioritized approach for development of specific protection against RVF. DNA vaccines encoding a respective target protein are one of the promising candidates. Immunization with a plasmid DNA containing an open reading frame with respective eukaryotic signal elements provides an in vivo synthesis of proteins whose conformational and post-translational modifications are identical to viral gene products expressed in course of the natural infection.

Based on the literature data and computer analysis of glycoproteins Gn, Gc and nucleoprotein N amino acid sequences predicted protective epitopes were determined using EpitPred [Imatdinov IR, 2012]. The selected sites were used to design to simulate an artificial polyepitopic protein by RaptorX and I-TASSER [http://raptorx.uchicago.edu; http://zhanglab.ccmb.med.umich.edu/I-TASSER]. As a result of this analysis, an amino acid sequence of a polypeptide variant «S73» which includes T -and B – lymphocytes epitopes was arranged (assembled).

In addition, native glycoproteins Gn and Gc of RVF virus under control of a cytomegalovirus promoter were cloned for comparative evaluation of an immune response to recombinant plasmids inoculation.

The Epizootic Hemorrhagic Disease Virus (EHDV) Induces and Benefits from Cell Stress, Autophagy and Apoptosis Title

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The mode and timing of virally-induced cell death withhold the potential of regulating viral yield, viral transmission and the severity of virally-induced disease. Orbiviruses such as the epizootic hemorrhagic disease virus (EHDV) are nonenveloped and cytolytic. To date, the death of cells infected with EHDV, the signal transduction pathways involved in this process and the consequence of their inhibition have yet to be characterized. Here, we report that the Ibaraki strain of EHDV2 (EHDV2-IBA) induces apoptosis, autophagy, a decrease in protein synthesis and the activation of c-Jun N-terminal kinase (JNK) and the phosphorylation of its substrate c-Jun. Inhibition of: (i) apoptosis with the pan-caspase inhibitor Q-VD-OPH, (ii) autophagy with 3-methyladenine or via the knockout of the autophagy regulator Atg5, and (iii) c-Jun phosphorylation with the JNK inhibitor SP600125 or with the cyclin-dependent-kinase (cdk) inhibitor roscovitine, independently attenuated the increase in viral titer in the course of infection. Moreover, SP600125 and roscovitine attenuated the EHDV2-IBA-mediated induction of autophagy. Taken together, our results imply that EHDV induces and benefits from the activation of signaling pathways involved in cell stress and death.

Identification of T cell epitopes in VP2 and NS1 proteins of African horse sickness virus in IFNAR$^{(-/-)}$ mice

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African horse sickness virus (AHSV) is an *Orbivirus* of the family *Reoviridae* that causes a severe disease in equids. AHSV virions are composed of seven structural proteins (VP1-VP7) arranged as three concentric layers surrounding the genome and four nonstructural proteins (NS1, NS2, NS3/3A, and NS4). Previous work in our laboratory showed the presence of AHSV-specific CD8+ T cells in mice immunized with recombinant Modified Vaccinia Ankara (rMVA) expressing VP2 and NS1 (1).
Using a combination of four epitope prediction algorithms (SYFPEITHI, BYMAS, NetMHC I and NetMHCpan) 4 peptides of VP2 and 5 peptides of NS1 from AHSV-4 were selected and synthesized. IFNAR(-/-) mice were immunized twice with rMVA expressing VP2 or NS1 proteins of AHSV-4 (10^7 pfu/mouse). Splenocytes of immunized animals were stimulated with 5 µg/ml of each peptide. ELISPOT and Intracellular Cytokine Staining (ICS) showed that VP2 (1052) peptide (YTFGNKFLL) and NS1 (92) peptide (CVIKNADYV) showed significant IFN-γ production in all the immunized mice and generated CTLs. These two peptides were identified as CD8+ T cell epitopes in AHSV-4 immunized mice. Furthermore, the IFN-γ production induced by VP2 (1052) peptide was similar to those induced by recombinant VP2 protein. In addition, this VP2 peptide is conserved among all AHSV serotypes. The identification of conserved T cell epitopes that induces CTL activity can contribute to the generation of protective multiserotype vaccines against AHSV.

(1) F. de la Poza, E. Calvo-Pinilla, E. López-Gil, A. Marín-López, F. Mateos, J. Castillo-Olivares, G. Lorenzo, J. Ortego. PLoS One 2013; 23;8(7):e70197.