Final report – KH 126521 research project

Different rotavirus species (RVA to RVJ) evolved to develop various effector functions to fight against the innate immune response of host cells. In case of RVA the product of genome segment 5, called non-structural protein 1 (NSP1), is a key element in this mechanism The NSP1 of RVA is a E3 ubiquitin ligase, which is able to inhibit virus multiplication at levels of pathogen pattern recognition, signal mechanisms through the intermediers of interferon (IFN) cascade and RNA transcription. Much less is known about the function (such as the possible anti-IFN effect) of NSP1 homologs of non-RVA (ie RVB to RVJ) strains whose sequence identity to their RVA counterparts may be as low as 10 to 20%.

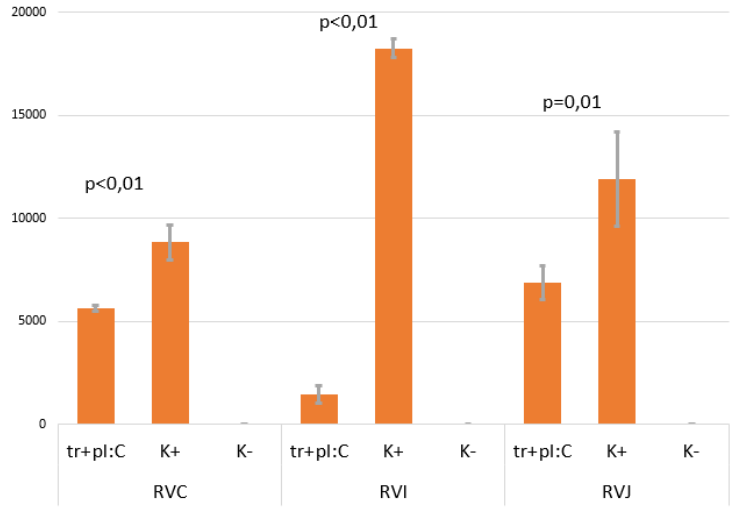
The KH-type grant scheme of the NKFIH (Nemzeti Kutatási, Fejlesztési és Innovációs Hivatal) allowed researchers to get a 2-year research funding on the basis of a single highly cited paper published recently. This key paper for our grant proposal reported the discovery and genetic characterization of RVI using viral metagenomics. Given that our research group has discovered two new rotavirus species in the past few years and described additional genetic diversity within other known non-RVA species (eg RVC) we felt that using the funding we could clarify some biological characteristics about these less known rotaviruses. Because these non-RVA strains are typically non-cultivatable we had to design molecular cloning and related *in vitro* experiments.

Our methodology included the following major steps. We amplified target genes by RT-PCR and cloned them into TA vector (pGEM-T) using heat-shock method in DH5-alpha cells. Then we subcloned the ORFs into pcDNA3 using the same method and the same bacteria. We used this eukaryotic expression vector (ie pcDNA3 based) to express cloned NSP1 homologs from RVC, RVI, and RVJ within cell (some other cloned inserts were not suitable for further analysis). To induce IFN we used poli I:C molecule. Numerous cells were used but some (eg Vero, MA104, BHK-21) were subsequently omitted from the work. The level of IFN expression was measured in other heterologous (ie poultry, porcine and feline origin) and homologous (ie canine origin) epithel cells (CRFK, MDCK, PK15, LMH) and fibroblast (A72) following transfection with NSP1 expressing vectors. Cells were maintained in DMEM with 7 or 10% FBS and 1% Penicillin/Streptomycin. For the experiments, 6-well plates were used and incubated in CO2 incubator. 24 h post transfection with plasmids, poli I:C was added (using various concentration ranges of poli I:C in different cell types) and after another 24 h incubation the IFN-beta specific mRNA was measured by using host species specific real-time RT-PCR methods; host species specific beta-actin (LMH) or GAPDH (CRFK, MDCK, PK15, A72) was also amplified as internal control that we used to determine the relative expression of target IFN gene. Although we planned to use interferon sensitive strains of RVA (as reporter virus) to infer the effect of cloned NSP1 homologs, we could not obtain such strains from international sources.

The results, in brief, were as follows. In CRFK and LMH cells we did not observe IFN-beta production following poli I:C treatment. In MDCK and PK15 cells we could measure IFN induction following poli I:C treatment, but the transfected NSP1 homologs did not result in reduction of IFN-beta gene expression. The canine origin A72 cell line was found as the single suitable model for our study. Although A72 represented a heterologous cell type for the bat-origin RVJ NSP1, a 40% of IFN reduction was measured when analyzing the effect of the RVJ NSP1 homolog. Furthermore, the canine RVC and the canine RVI NSP1 homologs also reduced IFN-beta expression by 36% and 83%, respectively (see Figure). Importantly, the RVI NSP1

coding genome segment encodes two ORFs (a smaller ORF at the 5'end and a larger ORF at the 3' end) and IFN expression decreased only when the two ORFs were co-expressed. This observation was unexpected given that recent research (doi: 10.1128/JVI.00813-19) predicted the smaller ORF, similar to the smaller ORF of RVB, to encode a syncytium forming protein. We, however, in our experiments did not observe this feature; instead this smaller ORF of RVI seemed to cooperate with the larger ORF to result in reduced expression of IFN-beta.

Figure. Relative amount of IFN-beta production in the presence and absence of expressed rotavirus NSP1 homologs



In our research plan we also planned to determine the cellular localization of NSP1 homologs and to do this we obtained immune sera generated against peptides that represented the most immunogenic epitopes; however, these antibodies apparently did not work perhaps because the predicted peptide epitopes were not suitable to reach our goals.

In our ambitious research proposal, when we aimed at gaining insight into the possible function of genome segment 5 homolog(s), we also adapted methods new to our research group. In this regard, our team achieved some of the goals. Thus, we generated new data of the biological function of NSP1 homologs of canine RVC, canine RVI and bat RVJ strains. However, based on literature data available for RVA we expected more significant reduction in IFN expression in cell culture. Although at present we cannot explain the difference in the IFN reduction capacity between RVA and selected non-RVA NSP1 proteins, we assume that the experimental setup or the use of cell type (A72) that supported our experiments may have played a role in this. It is also possible that the primary function of the proteins encoded by genome segment 5 in various non-RVA species may differ from those functions assigned to the NSP1 of RVA or even if the function is shared the efficacy of this function may show differences among strains. This could, in part, also explain why non-RVA strains are so rarely identified in field, why they do not cause as severe disease as RVA do and why they are shed at lower quantity in feces than it is seen in case of RVAs.

At this point the PI of the research needs to confess that the time required for a study with no previous methodological experience has been largely underestimated. An unfortunate consequence is that the publication activity has not fully met the expectations of the grant type.

We presented our preliminary results on a conference traditionally organized at the Veterinary University in Budapest. Nonetheless, the presented data obtained fairly good feedback that encouraged us to continue our work on interferon antagonism. Despite the fact that it was not possible to publish a research paper about the currently available results of the work within the timeframe of the 2-year project, we learnt new methods and new ways of thinking that we will use in the future to extend the study and try to make the obtained results publishable in a scientific journal.