Deliverable Report

Deliverable No:	D26.1	**Delivery Month:**	24
Deliverable Title	Assess the use of two eukaryotic expression systems; microalgae and a protozoan (*Leishmania tarentolae*) for production of nodavirus capsid protein		
WP No:	26	**WP Lead beneficiary:**	P7. IMR
WP Title:	Fish Health – Atlantic halibut		
Task No:	26.1	**Task Lead beneficiary:**	P7. IMR
Task Title:	Production of VNN capsid protein		
Other beneficiaries:			
Status:	Delivered	**Expected month:**	24
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Other Scientists participating: Nerland, A.H. (IMR)

Objective: The objective of this Deliverable is to assess the two eukaryotic systems -microalgae and a protozoan (*L. tarentolae*)- for the production of nodavirus (Viral Neural Necrosis, VNN) capsid protein, to be used for the development of an oral vaccine for Atlantic halibut (*Hippoglossus hippoglossus*). We have worked on several alternatives for the expression of nodavirus capsid protein in this task and we have two vaccine candidates: a vaccine with recombinant nodavirus capsid protein expressed in *E. coli*, which previously has been shown to elicit protection in turbot (*Scophthalmus maximus*) and to induce an immune response in Atlantic halibut with a protective character. Recombinant capsid protein has also been expressed in tobacco plants, from which it can be isolated as virus like particles (VLP) for integration in a vaccine.

Background: Recombinant capsid protein from nodavirus expressed in *E. coli* has been shown to induce protection when formulated in a vaccine. However, bacterial cells do not glycosylate the expressed protein, as do higher eukaryotes. It might be that other expression systems (**Fig. 1**) may provide antigens more like the naive viral proteins as produced after viral infection. By expressing the capsid protein of nodavirus recombinantly in different systems it would be possible to find out if post-translational modifications might influence antigenicity and thereby its ability to induce protection when used as antigen in a vaccine.

Description:
The aim was to express the protein antigen in systems having different post-translational modifications and test if that could influence the antigenicity of the proteins. An already existing collaboration with a research group working with plants was working with microalgae. Hence, one of these systems was originally planned to be microalgae. However, at the time DIVERSIFY started, our collaborators had already started cloning the gene into tobacco plants for transitional expression, and thus tobacco plants were used instead of microalgae.

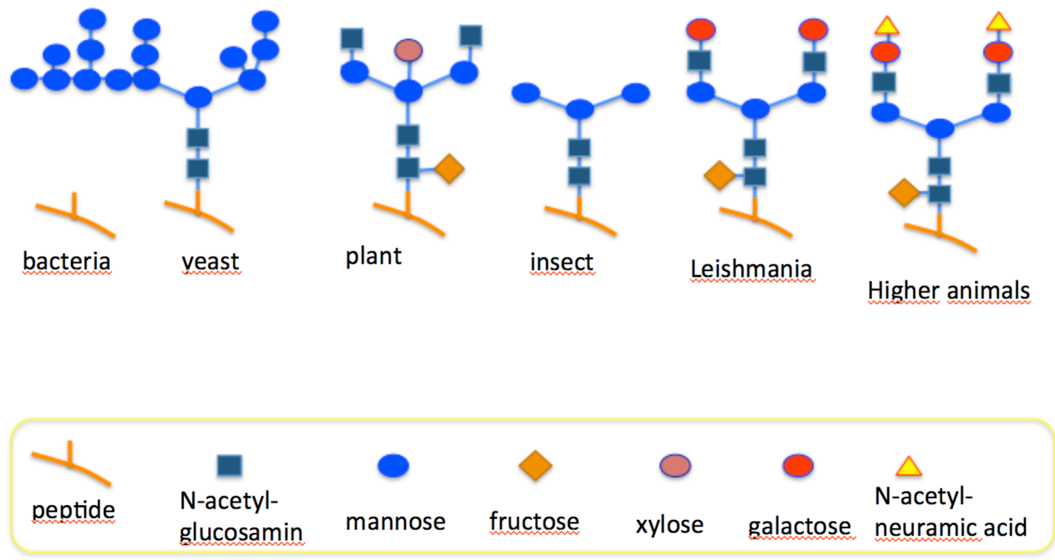

Figure 1. Glycosylation of proteins in different organisms, showing the extent of glycosylation.

Materials and methods:

1. **Expression in *E. coli***

The cloning and expression has been described previously (Sommerset et al., 2005). Briefly, total RNA was isolated from brain tissue of Atlantic halibut from a natural nodavirus outbreak, by the TriZol method. By using random primers and reverse synthetase cDNA was generated, which was cloned into the pCR-Script SK(+) vector (Stratagene, USA), giving a plasmid construct designated as pAHT6. Specific primers were then designed to clone the capsid gene into pET30b+ (Invitrogen), generating the plasmid pET30b (+) AHNVcapsid, where the capsid is expressed as a fusion protein with the His-Tag at the N-terminal end.

2. **Expression in *Leishmania tarentolae***

Using the pAHT6 as a template and with specific primers, a segment containing the capsid encoding sequence was generated by PCR and ligated into KpnI-NcoI digested vector pLexsy-sat2 (Jena Bioscience, Germany). This construct, designated as pLEXSYsat2AHNV(SJ)capsidHis6 was propagated in *E. coli*, isolated and digested with *Swa*I, generating a linear fragment where the capsid encoding sequence is flanked by the 5'ssu and the 3'ssu from the 18S gene of *Leishmania tarentolae*, respectively. Thus, the capsid gene was integrated in one of the 18s genes of the parasite. The linear fragment was transferred to *Leishmania tarentolae* by electroporation (BTXECM830, 6 times 300 micro-seconds pulses of 900V in a 2 mm cuvette). The protozoa were transferred to Lexsy BHI-medium (Jena Bioscience) and incubated overnight at 26 °C, then 100µg/ml NTC (Nourseothricin) was added and incubated for 48 h. Aliquots were spread on plates containing Lexsy BHI-medium added agar, and incubated at 26°C until colonies appeared (2-3 weeks). Single colonies were picked, propagated in Lexsy BHI-medium and frozen down. For expression, a clone was propagated in Lexsy BHI medium added NTC for around one week (10^7 cells/ml), harvested by centrifugation and tested for expression.

3. **Expression in tobacco plant**

Expression in tobacco plant was conducted at the John Innes Centre, Norwich Research Park, Norwich, UK. In brief, the same ORF coding for the capsid encoding sequence was cloned into the vector pEAQ-HT behind the CaMV promoter (Cauliflower mosaic virus) for transient expression in chloroplasts (Sainsbury et al., 2009), generating the construct pEAQ-HT-NNV-CP, which was transferred to tobacco plants by using *Agrobacterium tumefaciens*. This gives a transient expression in the leaves.

Western blot
To test for the expression in the different systems, samples were run on a 4-12 precast NUPAGE (Invitrogen), transferred to nitrocellulose filter using the iBlot Dry blotting system (Invitrogen), followed by the Western Breeze Immunodetection system (Invitrogen) using rabbit anti AHNV capsid antibodies (Sommerset et al., 2005) diluted 1:1000 as primary antibody.

Results and Discussion:
In all the three systems we got expression of recombinant capsid proteins. Recombinant proteins were isolated as inclusion bodies from the *E. coli* strain BL21 (DE3) after induction with IPTG. **Figure 2** shows a PAGE gel where serial dilutions of the recombinant protein expressed in *E. coli* were applied to elucidate the quality of the purification process. **Figures 3 and 4** show Western blots of samples from expression in *Leishmania tarentolae* and tobacco leaves, respectively.

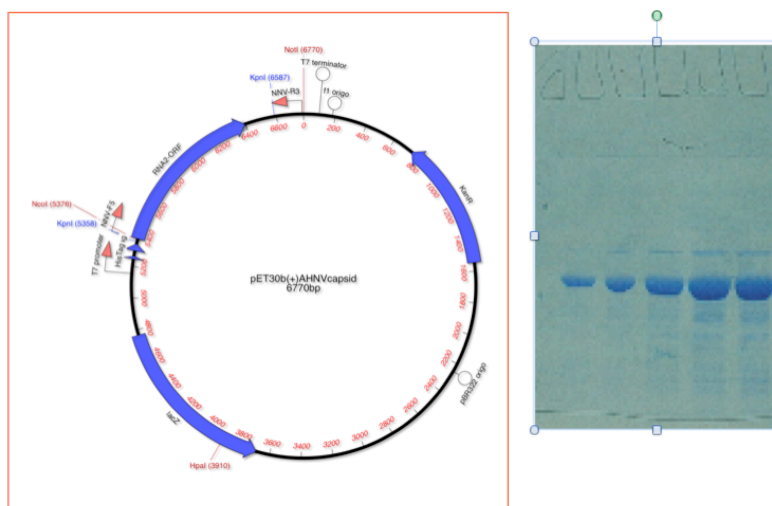

Figure 2. Expression in *E. coli*. Figure of the plasmid construct to the left. Picture of a 4-12 % Commassie Blue stained NuPage gel with 2x serial dilution of purified protein to the right.

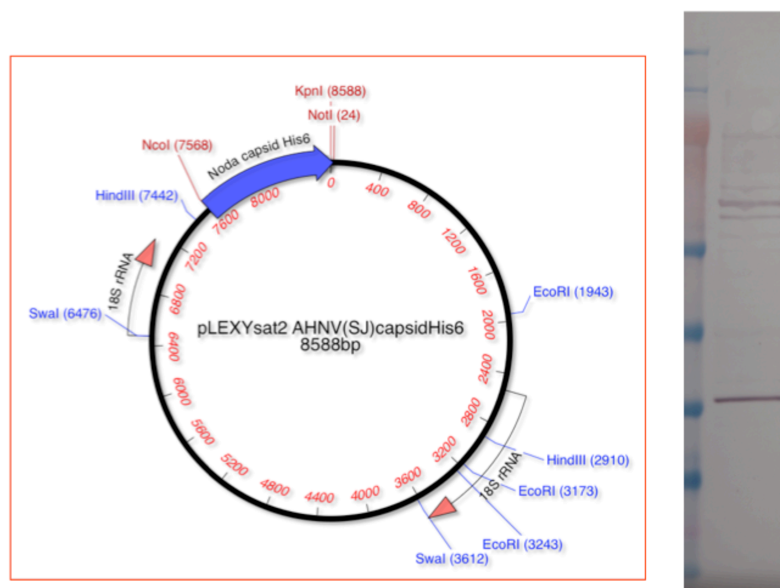

Figure 3. Expression in *Leishmania tarentolae*. The plasmid construct is shown to the left ,while to the right a Western blot showing expression of recombinant capsid protein is shown.

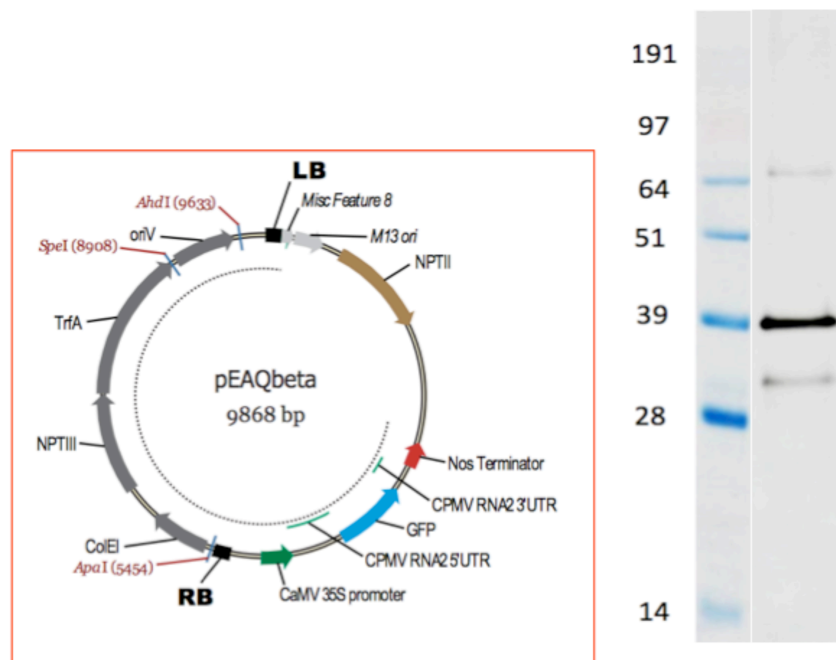

Figure 4. Expression in tobacco plants. Plasmid construct shown to the left, and to the right a Western blot showing expression of the capsid protein is shown.

Conclusion:
We have managed to express the nodavirus capsid protein in all three systems. However, it was only in the *E. coli* system that we achieved sufficient and high expression for further use of the protein as antigen for vaccination purposes. Further optimisation for sufficient expression in plant and protozoan systems and a method for purification of the recombinant protein still remain to be done. The initial testing of possible expression of VNN capsid protein was carried out using non-inducible systems, and the next step is to use an inducible expression kit to increase the production.

References:

Sainsbury, F., Thuenemann, E.C., and Lomonossoff, G.P. (2009). pEAQ: versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. Plant Biotechnol. J. *7*, 682–693.

Sommerset, I., Skern, R., Biering, E., Bleie, H., Fiksdal, I.U., Grove, S., and Nerland, A.H. (2005). Protection against Atlantic halibut nodavirus in turbot is induced by recombinant capsid protein vaccination but not following DNA vaccination. Fish Shellfish Immunol. *18*, 13–29.

Deviations: None

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