Final project report for:216177 - Studies of virulence mechanisms and host responses to infection with piscine myocarditis virus (PMCV)

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WP1 - Development of reverse genetics systems for PMCV

The full-genome PMCV sequence was synthesized by Aldevron, including an upstream T7 promoter for in vitro transcription. Furthermore, the sequence contained a hammerhead ribozyme sequence to ensure precise cleavage at the 5' end during transcription and a 3'end ribozyme for self-cleavage of the 3-end. The company found that the plasmid was unstable in the lab, prone to "falling apart" and they were unable to produce a stable clone. We obtained the material they had synthesized and transformed the plasmid into *E. coli* and managed to isolate a clone expressing the full-genome of PMCV in a pTurbo construct (reported ealier). The sequence of this plasmid was subsequently confirmed by re-sequencing.

Transfecting CHH-1 cells with PMCV full-genome under a CMV promoter

The rPMCV in pTurbo construct was transfected into CHH-1 cells using the Neon transfection system (Life Technologies), including controls. Real-time PCR confirmed that the PMCV genome was transcribed from the plasmid following transfection. High levels of transcripts were detected on day 1 and 2, however being slightly reduced at day 5. We were not able to detect expressed protein by immunostaining using the monoclonal mouse-anti ORF1 desc in CHH-1 cells transfected with the rPMCV in pTurbo construct.

In another study the cell morphology was monitored by phase-contrast microscopy following

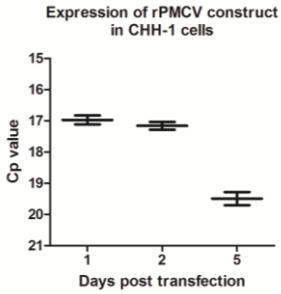


Figure 1: Real-time PCR confirmed the PMCV genome was expressed from the plasmid construct after transfection in CHH-1 cells.

transfection with rPMCV in pTurbo, pTurbo FP635N backbone (transfection control) and mock-transfected control cells. The results revealed that CHH-1 cells transfected with the full-genome PMCV construct displayed vacuoles in the cytoplasm at 14 days post transfection, while cytopathic effects (CPE) were not observed in mock-transfected cells and cells transfected with the pTurbo TP635N. Transfer of the supernatant to fresh cell cultures did however not result in similar vacuolization.

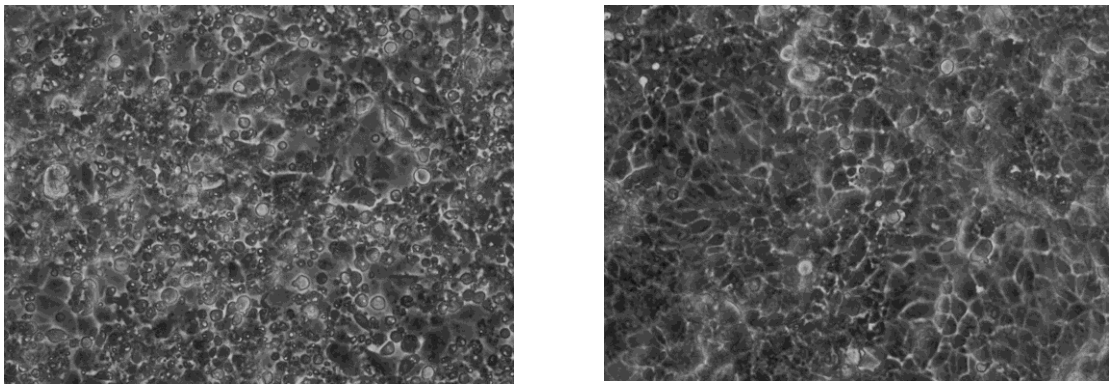


Figure 2: Cell morphology of CHH-1 cells transfected with rPMCV in pTurbo construct (left) and pTurbo backbone (right) at 14 days post transfection.

In vivo challenge experiment testing rPMCV in pTurbo and CMS positive tissue homogenate

Here we tested the infectivity of the PMCV full-length viral vector in vivo with a standard infection protocol using tissue homogenate. Cohabitants were included in the tank injected with the PMCV full-length viral vector to assess whether recovered virus (if any) were able to infect cohabitant fish horizontally during challenge. The experiment was done in freshwater at 10-11 ± 1°C in Atlantic salmon with an average weight of 34 gram. Histopathological changes of hear were assessed 6 and 12 weeks post injection and we measured viral load by real-time PCR in heart and head-kidney at 6 and 12 weeks post injection.

Some fish in all groups displayed minor histopathological changes consistent with CMS; more prominent at 6 weeks compared to 12 weeks.

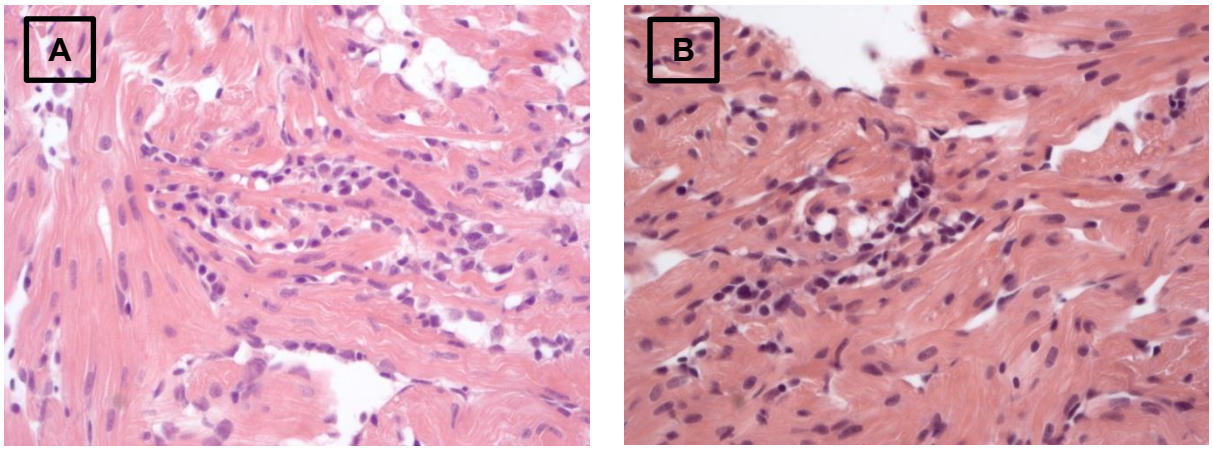


Figure 3: Histopathological changes in fish at 6 weeks. A: Fish challenged with rPMCV in pTurbo construct. B: Fish challenged with tissue homogenate from CMS diseased fish

Real-time PCR data confirmed infection with PMCV in fish challenged with infected tissue homogenate, and viral load in heart was higher at week 12 compared to week 6, while levels

in head-kidney peaked at 6 weeks post challenge. By real-time PCR the PMCV full-length viral vector was not able to "produce" replicating virus in plasmid injected fish (Fig. 4).

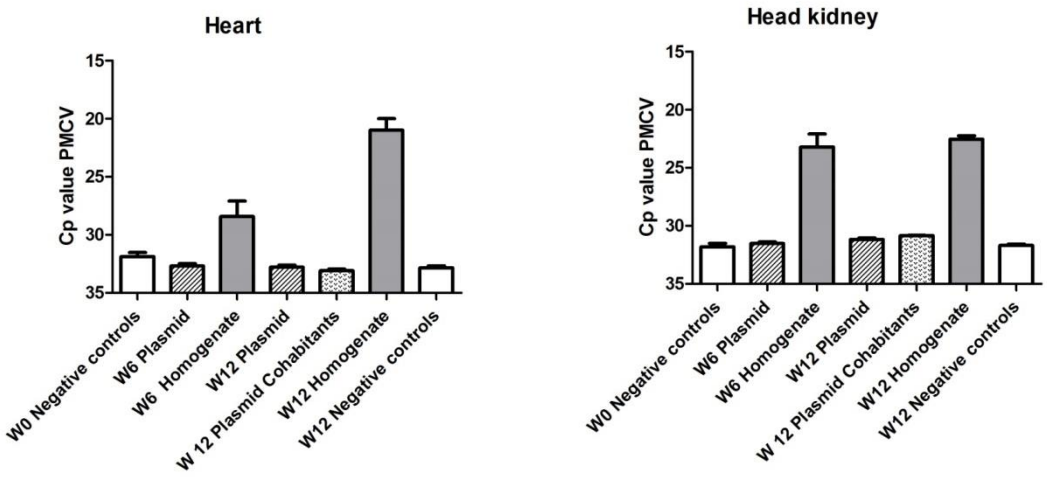


Figure 4: Real-time PCR data confirmed infection with PMCV in fish challenged with tissue homogenate, while fish injected with the full-genome viral clone were not detected (Cp values around 32 represent noise/non-specific signals).

WP 2 - Protein characterization

Recombinant expression of PMCV ORF encoded proteins. The three ORF's (capsid, polymerase and ORF3 which is unknown) were cloned into expression vectors with or without a green fluorescent tag in the both N-terminal and C-terminal end. Proteins with a C-terminal tag is used in the expression studies since the tag does not interfere with folding and protein function.

Both tagged and untagged proteins were all expressed in CHH-1 cells by transfection of cells using the expression plasmid and electroporating procedures with Neon transfection system. The cells were studied by phase contrast microscopy and fluorescence microscopy by time course studies after transfection/expression of proteins (Fig. 5).

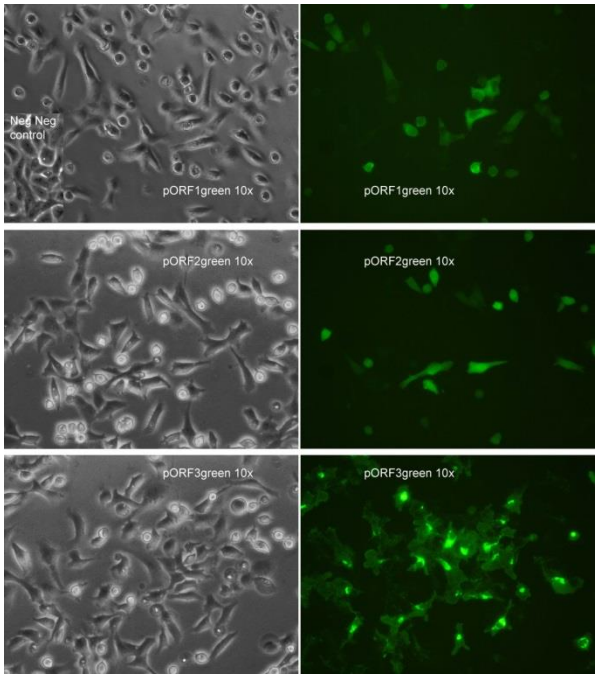


Figure 5. Two days post transfection

High expression efficiency is seen for all three proteins (fluorescent tag), as early as 6 hours post transfection and with an increasing number of cells expressing the proteins (i.e. amount of protein in cell) as the cells grow.

Immunostaining transfected CHH-1 cells

We purchased custom-made, monospecific virus peptide antibodies against proteins encoded by open reading frame 1, 2 and 3 using a commercial lab (Pacific Immunology). None of the obtained antisera showed reaction by western blots or when used to stain cells transfected with the three ORF's (both with and without a green fluorescent tag). This points to low immunogenicity or improper immunization protocols.

We also tested antibodies raised against ORF1 and 3 encoded proteins used to immunize rabbits (kindly provided by PHARMAQ). And we have tested monoclonal antibodies raised against ORF1. All "variant antisera/antibodies" were tested in transfected cells and in western blots. The monoclonal antibody and the polyclonal antibodies (rabbit) raised against ORF1 encoded protein (capsid) showed immunostaining of CHH-1 cells expressing ORF1. The monoclonal antibody had superior sensitivity and specificity compared to the polyclonal antibodies (Figs 6 and 7). The polyclonal ORF3-antibody gave no staining.

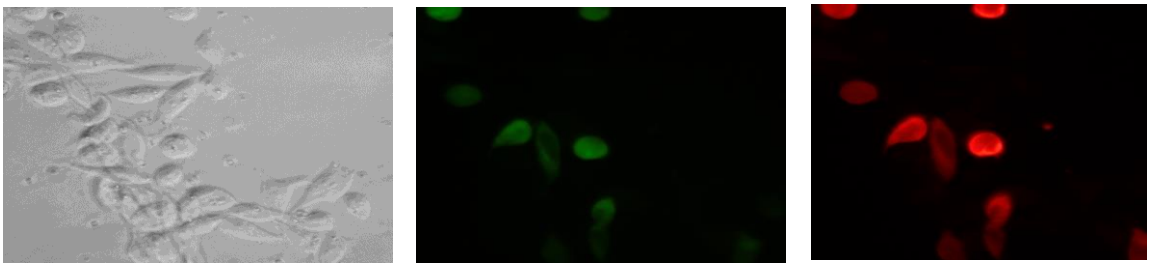


Figure 6: A monoclonal antibody against ORF1 protein successfully stained CHH-1 cells transfected with ORF1 tagged with GFP

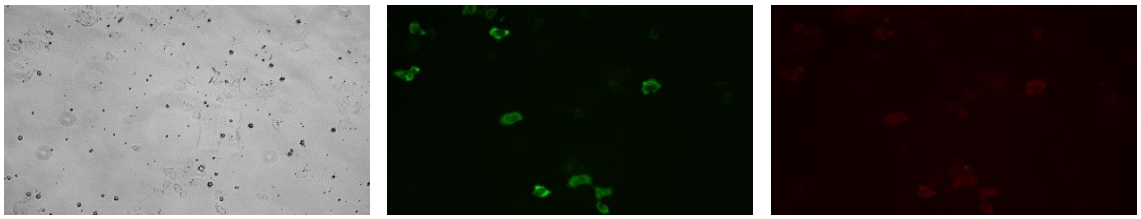
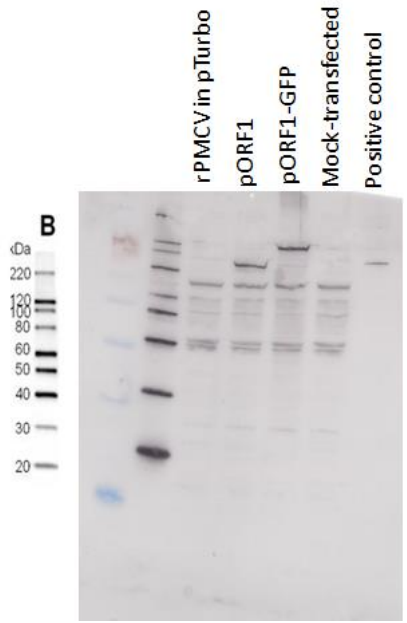


Figure 7: Polyclonal rabbit anti-ORF1 also stained CHH-1 cells transfected with ORF1-GFP

Detecting ORF1 and 3 encoded proteins by western blots



The same antibodies as described above were tested for reactivity to ORF1 and ORF3 proteins in western blots. Proteins were expressed in CHH-1 cells that were transfected with the ORF1 and 3 expression constructs (with and without GFP tag) followed by protein isolation using the CelLytic M – mammalian cell lysis/extraction agent (Sigma-Aldrich). Neither the monoclonal mouse-anti ORF1 antibody nor the polyclonal rabbit-anti ORF1

antibodies could detect the ORF1 protein in western blots (Fig. 8). The polyclonal ORF3-antibody was also negative (not shown).

Fig. 8. WB for ORF1 (pORF1) was negative. The pORF-1-GFP is positive and stained with an immune serum against GFP.

WP3 - Viral entry and release and WP 4 - Virus protein functional studies

Early studies of cells expressing the three proteins using confocal microscopy have been started and are under optimization of procedures. At the time, several antibodies against the ORF1-encoded protein are available (produced by PHARMAQ) and will be used in the planned confocal/standard microscopy studies on transfected/infected cells. The antibodies/fluorescence from tagged proteins will be used together with fluorescent markers against cellular compartments, which are under optimizing for use in confocal microscopy.

The ORF3 protein is unique in the sense that no proteins with similar sequence have ever been identified earlier (for all proteins available in the databank). Transfection studies with fluorescence tagged proteins show that the ORF1-protein (capsid) localizes to the cytoplasm of the cells while the ORF2-protein (polymerase) localizes throughout the cell (including nucleus). These findings are in line with the predicted type of protein.

The ORF3-encoded protein shows an extraordinary expression in the cells and with a cytotoxic effect. At early time points post transfection the protein is (likely) transported through the endoplasmic reticulum/Golgi (localized in spots around nucleus). At later time (2-3 days) the cells die with cell fragmentation, indistinct nuclei and indistinct cell borders, while the protein is still present in cell ghosts. No indication of apoptosis has been found.

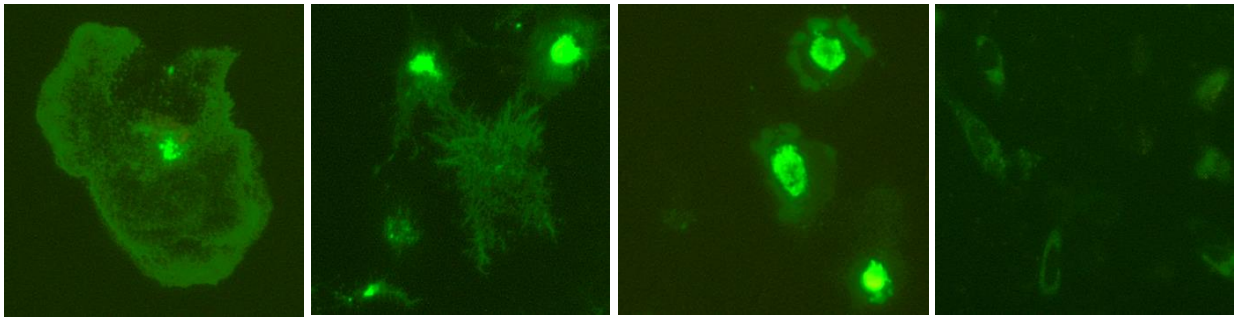
Since the virus cannot be grown consistently and reproducibly in cells the entry process and the release process remain poorly understood. Uptake mechanisms are not understood at all while the anticipation is that the release of virus from the infected cell in one way or another involve the ORF3 encoded protein, possibly through cell lysis. A very detailed analysis has been carried out with a focus on the ORF3 encoded protein.

Recombinant expression of PMCV encoded proteins in cultured CHH-1 cells

The ORF3 encoded protein is expressed with variable location and morphology, in perinuclear dense granules at early stage and staining spreads throughout the cell at later stages in a fiber-like expression pattern (Fig. 9) and cells die through a necrosis process.

Cell toxicity of PMCV ORF3 protein varies between piscine cells and mammalian cells

Piscine and mammalian cells in culture from several fish and mammalian species were used to test toxicity of ORF3 protein, and CHSE and EPC cells resembled CHH-1 cells. CHO cells (from hamster) did not show indication of toxicity (Fig. 9).



CHSE (salmonid) EPC (carp) AGK (grouper) CHO (hamster)

Fig. 9. Toxicity in different cell lines as indicated.

ORF3 protein aggregates into polymers held together by disulfide bonds

The wtORF3green protein is seen as aggregations with globular morphology with dense fiber-like protrusions. To verify to what extent disulfide bonds were involved in aggregation, proteins were analyzed by SDS-PAGE under reducing and non-reducing conditions, the latter keeping disulfide bonds intact. ORF3 with tag in C-terminal and N-terminal end showed a smear stretching from appr. 100kDa and up to start of lanes, which indicates that ORF3 is prone to aggregate into polymers of a wide range of sizes from 100kDa and up to more than 300kDa, held together by disulfide bonds. The control protein ORF1green showed only one product band representing full length protein under both conditions.

ORF3 protein promotes cell lysis and virus propagation

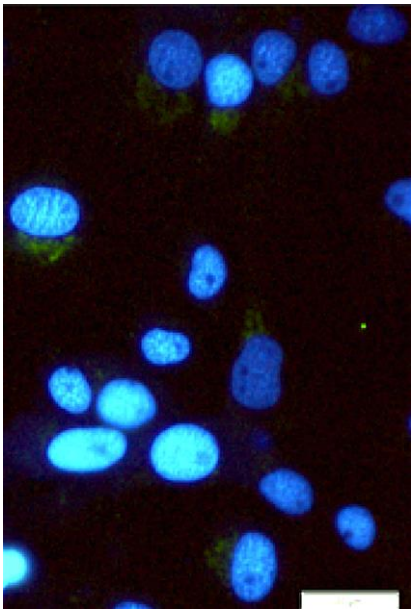


Fig. 10. Green fluorescent shows virus presence in EPC cells

Since we are not able to consistently grow PMCV in cell culture it is difficult to assess the importance and function of the ORF3 protein in relation to a virus infection. We therefore took an alternative approach a piscine rhabdovirus, viral hemorrhagic septicemia virus (VHSV) to test this. We have developed a reverse genetics system for VHSV (through a different project) and generated a hybrid virus consisting of the genome backbone of an attenuated VHSV (attVHSV) plus the ORF3 gene was cloned into the genome of VHSV as a “foreign gene”, with a C-terminal His-tag, termed attVHSV-ORF3His. First, the expression of ORF3 was verified in infected EPC cells by immunofluorescent staining on fixed cells against the His-tag (Fig. 10).

The ORF3-protein was confirmed to be expressed by the hybrid virus and we then compared the growth characteristics of the hybrid virus strain - attVHSV-ORF3His – and showed that this strain restored the virus' ability to cause CPE in the cell cultures comparable to wtVHSV, at 2dpi at 15ºC (Fig. 11). The control infection using attVHSV resulted in no CPE.

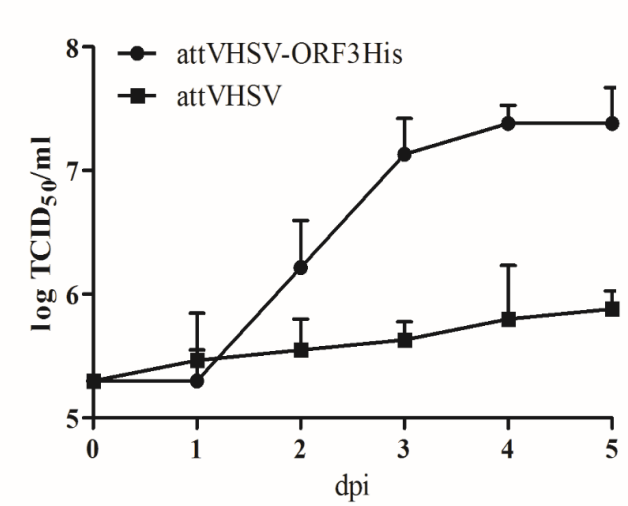


Fig. 11. Growth characteristics of attVHSV-ORF3 and attVHSV.

WP 4 - Virus protein functional studies

The hemagglutination ability of PMCV was tested using a standard assay with tissue and supernatant from infected cells (with known high concentration of virus tested with Real Time PCR) and red blood cells of Atlantic salmon. No hemagglutinating effect was observed.

WP5 - Studies of host responses and risk factors

A. *Effects of stress-responsiveness.*
B. *B. Effect of suboptimal oxygen levels.*
C. *C. Effect of co-infection with PRV.*

Stress responders

Experimental groups to test in the challenge trial were produced at Nofima, Sunndalsøra spring/summer 2013. This followed the approach of selecting fish according to swimming endurance capacity of different groups of salmon exposed to increasing water current conditions. These groups were selected using a new state-of-the-art swimming flume device (Takle/Jørgensen, internal Nofima project), facilitating controlled water speed, high reproducibility and reduced fish handling. Consequently, improved experimental groups with contrasting phenotypic traits of swimming capacity will serve as a criterion of selection for inclusion into the challenge experiment using PMCV.

Swim groups were further compared in closed respirometry tests measuring oxygen consumption (MO_2) during 2 hrs after swimming to critical speed (Ucrit). This showed that good swimmers in contrast to poor swimmers had numerically lower MO_2 kg/h during the first 6o min, which was concordant with a significant lower expression of stress-related genes. These results may indicate an association between swimming capacity and aerobic and stress-coping performance, which will be further evaluated during the PMCV infection trial.

Suboptimal oxygen levels

Evaluation of pathogenesis during the CMS challenge trial with or without fluctuating hypoxia treatment (5-9 weeks post challenge, wpc) revealed three interesting observations.

1) During the later stage of CMS, PRV starts to replicate and contributes to increase the overall pathology by inflammation in epicardium.

2) Hypoxia treatment resulted in elevated heart pathology score (atrium + ventricle) by 11 wpc, indicating a delayed recovery from CMS.

3) Hypoxia gave elevated PRV load in heart by 11 wpc.

To understand better the molecular mechanisms behind these effects we are now analyzing PRV levels and various gene markers for oxygen transport and their expression levels in erythrocytes (with qPCR) from infected groups with and without hypoxia. We will also analyse erythrocyte PMCV levels in the same samples for better understanding of the co-infection dynamics between CMS and HSMI in blood.