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Evaluation of the gene expression of STING, IFN- β and osteopontin in tissue obtained from pigs treated with Matrix-M

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Independent project in Biology, EX0542 15hp, A2E
Biotechnology – Master's Programme



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Effekt av Matrix-M på uttryck av generna för STING, IFN- β , och
osteopontin i vävnad från gris

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SUMMARY

Matrix-M is the adjuvant component in traditional ISCOM vaccines. The adjuvant when used along with antigen increases the efficiency of the vaccines by inducing a balanced Th1/Th2 response and a long-lasting antibody response. Transcriptomal studies in the pig show that the majority of upregulated genes during the early inflammatory response to Matrix-M alone are Interferon regulated genes (Ahlberg, Lovgren Bengtsson, Wallgren, & Fossum, 2012). The present study aimed to establish qPCR assays for detection of gene expression of stimulator of interferon genes (STING), osteopontin (OPN) and interferon-beta (IFN- β) in porcine tissue. STING is a protein localized in the endoplasmatic reticulum that on activation is believed to enhance the production of Type I interferons (Sun et al., 2009). Overexpression of OPN is believed to play a key role in inducing the production of Interferon- α and other Type 1 interferons by plasmacytoid dendritic cells (Shinohara et al., 2006). A comparative gene expression study of STING and OPN in pigs treated with Matrix-M revealed that one or both of the genes were up-regulated in pigs with increased expression of the IFN- β gene 24 hours after administration of Matrix-M.

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Abbreviation

IFN- β	- Interferon Beta
OPN	-Osteopontin
PRR	-Pattern Recognition Receptor
PAMP	-Pathogen Associated Molecular Pattern
STING	- Stimulator of Interferon Genes
ISCOM	-ImmunoStimulating Complex
NK cells	- Natural Killer cells
qRT PCR	-Quantitative Real Time Polymerase Chain Reaction
GAPDH	- Glyceraldehyde 3-phosphate dehydrogenase
RPL32	- Ribosomal protein L32

1. Introduction

When pathogens invade the host, innate immunity or non-specific immunity is the first line of defense acting against them. The host system can act against the pathogens only if they recognize them. This is accomplished by pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs) (Palm et al., 2009) that recognize the microorganism. In case of virus's, the viral nuclei acid acts as a pathogen-associated molecular pattern which is detected by the PRRs (Olagnier & Hiscott, 2012). The innate immune response is initiated via the PRRs. Predominantly in virus recognition the two main PRRs groups involved are the Toll-like receptors and the RNA helicases RIG-I, LGP-2 and MDA5 (Yoneyama et al., 2005). Various signaling pathways are involved in the activation of the host defense mechanism. Activation of some signaling pathways leads to the expression of Type I interferons (Palm et al., 2009).

1.1 STING

Recently it has been observed that an adaptor known as Stimulator of interferon genes (STING) leads to the expression of Type I interferons and Interferon Stimulated Genes (ISG) by activating a signaling pathway (Sun et al., 2009). STING is a transmembrane protein localized in the endoplasmatic reticulum and is found in various cell types such as endothelial cells, dendritic cells and macrophages (Barber, 2011). STING is also known as MPYS/MITA/ERIS/TMEM173. The entire reading frame of STING in *Sus scrofa* (Pig) is 1137 bp in length. The entire gene is composed of 378 amino acids. Real time qPCR analysis carried out in Tibetan Pigs showed that overexpression of STING led to up-regulation of IFN- β and IFN-Stimulated gene 15 (ISG15) (Huang et al., 2012). Studies carried out by Holm et al on various adaptors showed that the fusion of viral envelope with cellular membrane can mediate the recognition by the innate immune system (Olagnier & Hiscott, 2012). This leads to the production of Type I interferons and ISGs by the activation of a STING dependent signaling pathway. Mouse Embryonic Fibroblast collected from mice lacking STING were deficient in the production of Type I interferons when infected with HSV-I (Ishikawa, Ma, & Barber, 2009).

1.2 Osteopontin

Osteopontin is a negatively charged acidic hydrophilic protein and multifunctional in nature. OPN is also called as secreted phosphoprotein 1, bone sialoprotein, early T lymphocyte activation 1 protein, urinary stone protein, uropontin and nephropontin (Cao & Liu, 2006). Osteopontin is encoded by the gene *Spp1*. The mammalian OPN is made up of 300 amino acid residues (Mazzali et al., 2002). Expression of OPN is observed in various cell types such as macrophages, endothelial and epithelial cells. OPN is secreted into various body fluids and prior to its secretion, it is believed to go through post transcriptional changes such as phosphorylation and glycosylation (Cao & Liu, 2006). In case of a viral or bacterial infection, expression of OPN is observed in activated T cells DCs and macrophages. Improper or down regulation of OPN expression resulted in various autoimmune diseases such as lupus nephritis, multiple sclerosis and rheumatoid arthritis (Shinohara et al., 2006). Overexpression of OPN is believed to play a key role in inducing the production of Interferon- α and other Type 1 interferons by plasmacytoid dendritic cells. Studies carried out on *Spp1* deficient mice showed that OPN favored Type I immune response by expressing Interleukin 12 and suppressing Interleukin 10 (Shinohara et al., 2006).

1.3 IFN- β

Type I interferons (IFN α/β) are cytokines with an antiviral activity. These interferons possess anti-inflammatory properties. IFN- β plays an effective role as regulating agent by upregulating the expression of anti-inflammatory agents and by downregulating the expression of proinflammatory cytokines (Kieseier, June 2011). A recent review by Levy et. al suggests that IFN- β expression is induced by STING and OPN (Levy, Marié, & Durbin, 2011). OPN and STING are believed to act through two different pathways to induce the expression of IFN- β .

1.4 ISCOM Matrix

The immunostimulating complex (ISCOM) is a vaccine concept that was invented by Professor Bror Morien at the Swedish University of Agricultural Sciences. The adjuvant component in ISCOM vaccines, Matrix-M, induces contains purified saponin fractions, cholesterol and phospholipids (Morein et al., 1984).

Adjuvants play an important role in increasing the efficiency of the vaccines. This ISCOM Matrix-M is used with multimeric antigen that increases the antigens cross presentation activity of the dendritic cells by interacting with them (Maaskovsky et al., 2009). This in turn increases the immune response. When ISCOM matrix was injected in mice without crosslinking antigen, protective immunity was observed. From this observation, it is believed that ISCOM Matrix-M in itself has an immunomodulatory function (Wilson et al., 2011). At the site of administration of Matrix-M a rapid influx of neutrophils, monocytes, NK cells, B Cells, T Cells and Dendritic Cells was observed (Duewell et al., 2011; Wilson et al., 2011). Studies in the pig show early transcriptional response to the adjuvant. The majority of the up-regulated genes during this transcriptional response were interferon regulated (Ahlberg et al., 2012) Therefore, it was hypothesized that Matrix-M caused STING activation.

My project focuses on gene expression studies related to STING in various lymph nodes obtained from ISCOM treated and untreated pigs. This comparative expression studies using qPCR will help us to understand the activation of interferon-regulated genes in Matrix-M treated pigs.

1.5 qRT-PCR

In biological research, gene expression studies are gaining increasing importance. Understanding the expression of different genes in different tissues provides us an insight of the regulatory networks (Hellemans, Mortier, De Paepe, Speleman, & Vandesompele, 2007). Also, it helps to find out which genes that might play regulatory roles in complex diseases. Gene expression studies can be carried out through q RT-PCR technique. It is possible to simultaneously measure the expression of a gene in different samples. Q RT-PCR has an advantage over other conventional methods such as northern-blot and ribonuclease protection assay in speed, automation and throughput (Vandesompele et al., 2002). While performing a qPCR study it is necessary to normalize the mRNA fraction. This can be done with the help of internal control genes or housekeeping genes. Housekeeping genes considered for normalization should not vary in gene expression in response

to the experimental treatments. Commonly used housekeeping genes include GAPDH, β -actin, YWHAZ, HMBS, HPRT, RPL13A, UBC, and TBP (Vandesompele et al., 2002). High-gene specific variations has been observed in studies with one control gene. Hence, it is advisable to use more than one housekeeping gene while performing gene expression studies. The house keeping genes used in my study were RPL32 and YWHAZ gene.

2. Materials & Methods

2.1 Sample

Archived material obtained from twelve pigs in two experimental groups was used. Each group comprised 6 pathogen free castrated male pigs. The pigs numbered from 1 to 6 had been injected with 150 μ g Matrix-M (AbISCO 100; Isconova; Uppsala, Sweden) suspended in 1 ml sterile endotoxin-free 0.9 % NaCl solution. Pigs numbered from 7 to 12 received only saline. Tissue sample from the draining lymph node were collected from these two groups of pigs 24 hours after injection. The mRNA isolation and cDNA synthesis was carried out by Viktor Ahlberg prior to the commencement of the study.

2.2 Optimization of RPL32, YWHAZ, OPN & STING primers

Forward and reverse primers were designed for YWHAZ, OPN & STING genes. The primers for RPL 32 were already available. Some primers were picked from the literatures as specified in Table 1. The lyophilized primers were diluted to a concentration of 10nM in [distilled water?](#). An assay was developed using lab reference cDNA as the template. In the PCR room, to prevent contamination, the Master Mix was prepared in Master Mix hood and the template was exclusively handled in the template hood.

For each reaction, 1 μ l of lab reference cDNA was added along with SYBR green mix, forward primer, reverse primer and water to reach a 25 μ l reaction volume. Samples were amplified in duplicates in an iQTM5 real-time PCR detection system (Bio-Rad; Hercules, CA) with an initial step at 95^o C for 15 minutes and 40 cycles at 95^o C for 15 seconds and 60^o C for 30 seconds. The SYBR green fluorescence was measured at 60^o C for each cycle. A duplicate of negative control with no template was included at each assay. The melt curve started at 60^o C and ended at 95^o C at an interval of 0.5^o C and a dwelling time of 10 seconds.

The amplified PCR products were purified using E.Z.N.ATM Gel Extraction kit. During the first step of the purification process, agarose gel electrophoresis was performed on the amplified PCR products to fractionate the DNA fragments. It is highly recommended to use fresh TBE buffer. When the bands were separated, they were excised carefully under the UV illuminator. The excision of bands should be carried out fast to avoid over exposure to UV rays. If any nonspecific binding took place, additional bands could be observed. The excised bands were subjected to various treatments using Binding buffer, SPW wash buffer and elution buffer as per the steps mentioned in the E.Z.N.ATM Gel extraction protocol. The eluted purified PCR product was stored at -20^o C.

The copy number of the transcripts was roughly calculated. From the purified products and based on their copy numbers, dilution series starting with 10⁸ to 10⁰ was prepared. An assay was set up with 1 μ l of the purified products as the template along with SYBR green mix, respective forward

and reverse primer, and water to 25 µl reaction volume. Samples were amplified in duplicates in an iQ™5 real-time PCR detection system as described above.

The annealing temperature and concentration of the primers have to be optimized. These are the most important parameters for reaction specificity. When the annealing temperature is set too low it may lead to amplification of nonspecific PCR products. Similarly, setting high annealing temperatures may reduce the yield of the PCR product. This makes optimization of annealing temperature and concentration of the primer essential and a gradient check was carried for YWHAZ, OPN & STING primers to find out their optimal annealing temperature and optimal concentration. The iQ™5 real-time PCR detection system has the thermal gradient feature which allows to check a range of temperatures consecutively. While performing dilution series, it is important to check the primers for them to be considered efficient. The efficiency value shown by iQ™5 real-time PCR detection system (Bio-Rad; Hercules, CA) should be more than 90 % , slope between -3.3 to -3.6 and the R² value above 0.99 is preferred.

2.3 Relative gene expression analysis of STING and Osteopontin in Matrix-M treated and untreated pigs

A qPCR assay was set up with the cDNA sample from the pigs treated with Matrix-M (1-6) and untreated pigs (7-12). Four different assays were set up with STING, OPN, RPL32 and YWHAZ. The samples were run in duplicates along with negative controls and positive controls. The positive control was the cDNA templates from the dilution series of the purified PCR products. Water from the SYBR green kit was used as the negative control.

2.4 Analysing data from Realtime qPCR result

From the gene expression result, the data analysis was calculated based on Livak/Schmittgen method, Pfaffl method and Vandesompele method. The data from the real time PCR can be analyzed in two different ways, by absolute quantification or by relative quantification. Absolute quantification is performed in studies where determination of the exact copy number of transcripts is necessary. It is calculated based on internal or an external calibration curve. Relative quantification illustrates the change in expression of the target gene to that of one or more reference or housekeeping gene. Using Livak/Schmittgen method, we calculated the fold change using $2^{-\Delta\Delta Ct}$ values. First, normalization to the reference gene was carried out (ΔCt) which was done by subtracting the Ct values of reference genes to the Ct values of the target gene. Then, the average untreated control is used as the calibrator value to calculate ΔCt calibrator values and the fold change values ($2^{-\Delta\Delta Ct}$ values). Using the Livak/Schmittgen method, the entire calculation is done with an assumption that the amplification efficiencies of both the reference and target gene are equal (Livak & Schmittgen, 2001). In Pfaffl and Vandesompele method, the calibration of the Ct values to that of the calibrator was done first. The average Ct values of the untreated control pigs were used as the calibrator, and the ΔCt calibrator values of the reference genes (RPL32 and YWHAZ) and target genes were calculated. Later, normalization to the reference gene is carried out (Pfaffl, 2001). In Vandesompele method, first the ΔCt calibrator values of the reference gene and target gene were calculated with the average Ct values of untreated pigs as the calibrator value. The influence of different efficiencies on the fold change was calculated, followed by normalization to the geometric average of the reference gene (Vandesompele et al., 2002). In both

Vandelsompele and Pfaff method, the individual amplification efficiency of the reference and target genes was considered and calculations were made based on the efficiencies.

2.5 Primer selection

The primer pairs used for the three genes of interest and the two housekeeping genes were selected from different articles (listed in Table 1). As far as possible, designs spanning an intron were chosen. The primer sequences, their positions on the reference file used (also listed), the expected size of the PCR product on mRNA and genomic DNA, and the presence of introns are summarized in Table 1.

Table 1. Primer Information

Gene	Primers (Nucleotide position)	mRNA file	Expected size of PCR product	Intron spanning	PCR product size on genomic DNA	Reference
STING	TTACATCGGGTACCTGCGGC (489-508) CCGAGTACGTTCTGTGGCG (572-553)	NM_00114283 8.1	84bp	Yes	2932bp	Xie et al., 2010
RPL32	CGGAAGTTTCTGGTACACAATGTAA (249-273) TGGAAGAGACGTTGTGAGCAA (342-322)	NM_00100163 6.1	94bp	Yes	2765bp	Dawson et al., 2005
YWHAZ	ATTGGGTCTGGCCCTTAAC (961-980) GCGTGCTGCTTTGTATGACTC (1106-1085)	XM_00192722 8.4	146bp	Yes	227bp	Uddin et al., 2011
OPN	TTGGACAGCCAAGAGAAGGACAGT (731-754) GCTCATTGCTCCCATCATAGGTCTTG (851-826)	NM_214023.1	121bp	No	121bp	White et al. 2005
IFN- β	TAGCACTGGCTGGAATGAAACC (488-509) TCAGGTGAAGAATGGTCATGTCT (427-405)	NM_00100392 3.1	140bp	No	140bp	Lin et al., 2013

*On mRNA file listed.

3. Results and Discussion

3.1 Optimization of assays

3.1.1 Production of template for PCR optimization

The primers were checked for their specificity and the assay conditions were optimized using a lab referece cDNA. The amplicons obtained were run on the gel electrophoresis and to check their quality and to find out whether the obtained PCR product is of expected length. The expected PCR product length of STING primer was 84 bp. In accordance, Figure 1 shows the presence of STING band at 84bp position with respect to the 50bp ladders used. Once the gene electrophoresis was

completed, the bands were excised from the gel and we proceeded with the purification step using E.Z.N.A™ Gel extraction protocol. In a similar fashion, PCR templates of IFN- β , OPN, RPL32 and YWHAZ were obtained and ran on the gel.

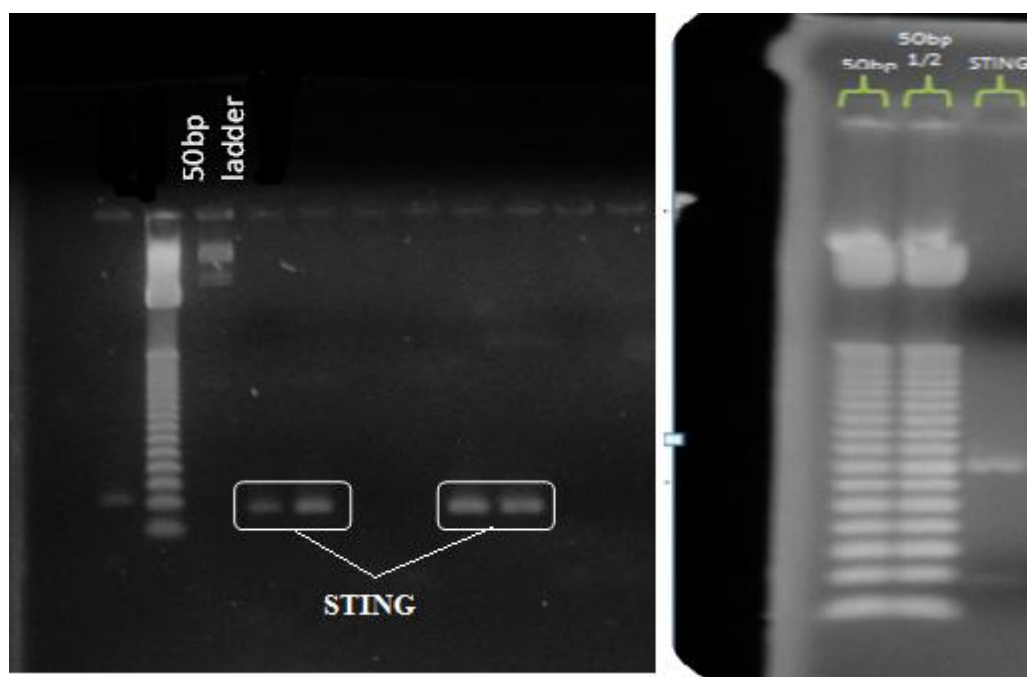


Figure 1: Gel Electrophoresis-STING primer PCR product

The purified amplicons concentrations were measured and diluted to 10^7 copy number dilution. Then dilution series of the STING amplicons were carried out to determine the end point and the appropriate dilution that can be used to carry out the gradient test. Dilution check was carried out from 10^6 to 10^0 dilutions. Amplification was detected in all the dilutions used. The 10^1 dilution was considered to be the best to proceed with the gradient test as the Cq value of the particular dilution was close to 25 cycles. In the case of OPN, the dilution series used ranged from 10^8 to 10^0 . Only first four dilutions were amplified and consequently the 10^6 dilution was considered to be appropriate to perform the gradient test.

3.1.2 Gradient test

In general, the assay was most efficient at 500nM primer concentration, resulting in earlier Cq values, when compared to 300nM and 400nM (Fig. 3). From the melting curve analysis (Fig. 2b) it was clear that the primer specificity was good, i.e. there was no evidence of primer dimer formation or other PCR artefact present. The melting point of the STING amplicon was at 82°C .

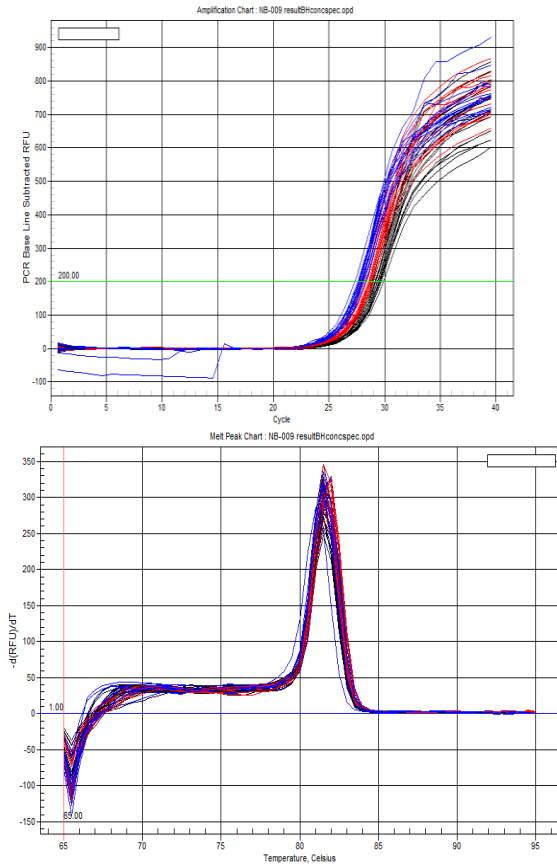


Figure 2 (a and b): Amplification Curve-STING & Melting Curve- STING (Purified PCR product)

Evaluation of the temperature gradient suggested an optimal annealing temperature around 54-58°C with primer concentrations around 400-500nM (Fig.3). A combination of 500nM primer concentration and annealing temperature of 56°C was chosen for further evaluation on serially diluted targets.

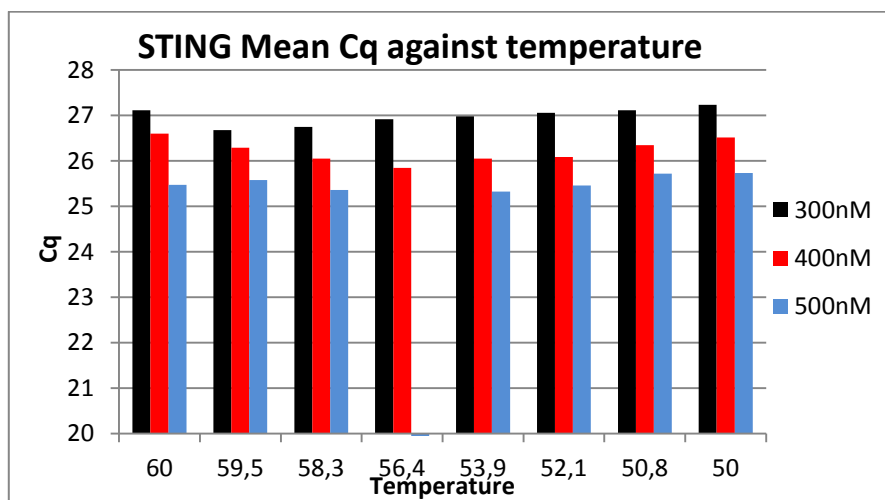


Figure 3: Cq values at different temperatures and Primer Concentrations

3.1.3 Confirmation of optimized conditions on serial diluted templates

The optimal condition of the STING assay was further validated on serial dilutions of both the STING amplicon (data not shown) and porcine cDNA (Fig. 4). Both amplification curves and melt curve analysis on serial dilutions of porcine cDNA indicated good specificity and no influence of primer dimers.

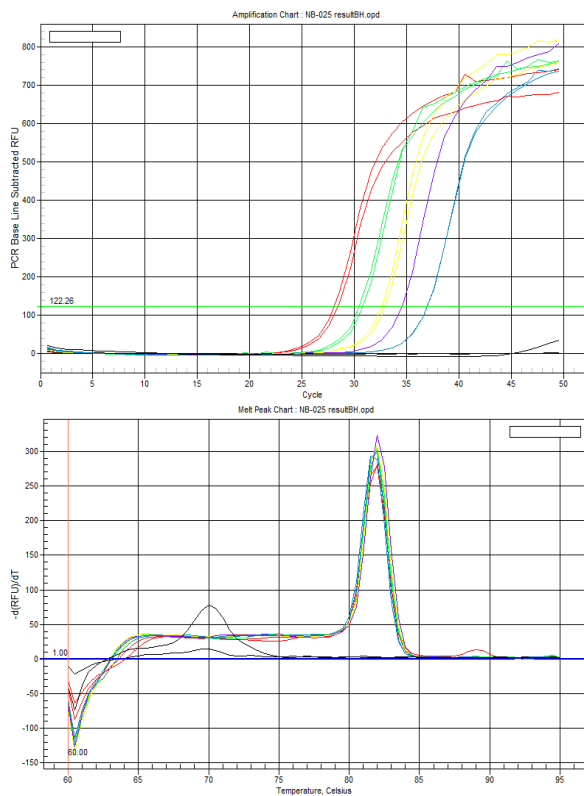


Figure 4 (a and b): Amplification Curve-STING & Melting Curve- STING (cDNA)

An acceptable run should have a slope within -3.3 to -3.6, an R^2 above 0.990 and a PCR efficiency above 90% (Ponton et al., 2011). The STING assay on porcine cDNA had a slope of -3.54, an R^2 value of 0.997, and a PCR efficiency of 90% (Fig. 5), fulfilling the criteria for a successful optimization.

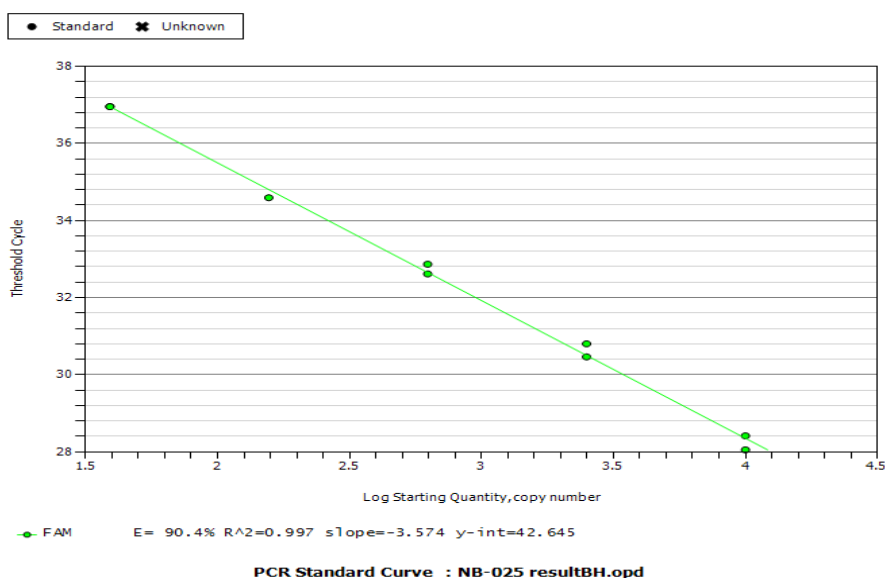


Figure 5: PCR standard slope curve-STING assay on porcine cDNA

3.1.4 Optimized results

The same optimization procedures, exemplified by the STING assay, was also applied to porcine IFN- β , OPN, RPL32 and YWHAZ, in collaboration with or by colleagues at the department. The results of the optimization for all assays are summarized in Table 2.

Table 2: Optimized conditions of genes of interest and housekeeping genes

Gene	Optimal Temperature	Optimal Concentration	PCR efficiency	R ²	Melt point, °C	No. of (1:4) dilution points
IFN- β	58°C	400nM	104%	0.962	79-79.5	5
OPN	56°C	300nM	93%	0.997	82.5	6
RPL32	55°C	300nM	97%	0.997	77	5
STING	56°C	500nM	90%	0.997	82	5
YWHAZ	58°C	400nM	92%	0.986	78.5	5

3.2 Gene Expression Analysis in lymphoid tissue

Data was analysed and calculated with the Cq values obtained from the assay on Porcine cDNA lymph node samples 1-6 (Matrix-M treated) and 7-12 (mock treated). Fold change was calculated based on three different methods Livak/Schmittgen 2001, Pfaffl 2001 and Vandesompele 2007 method.

STING

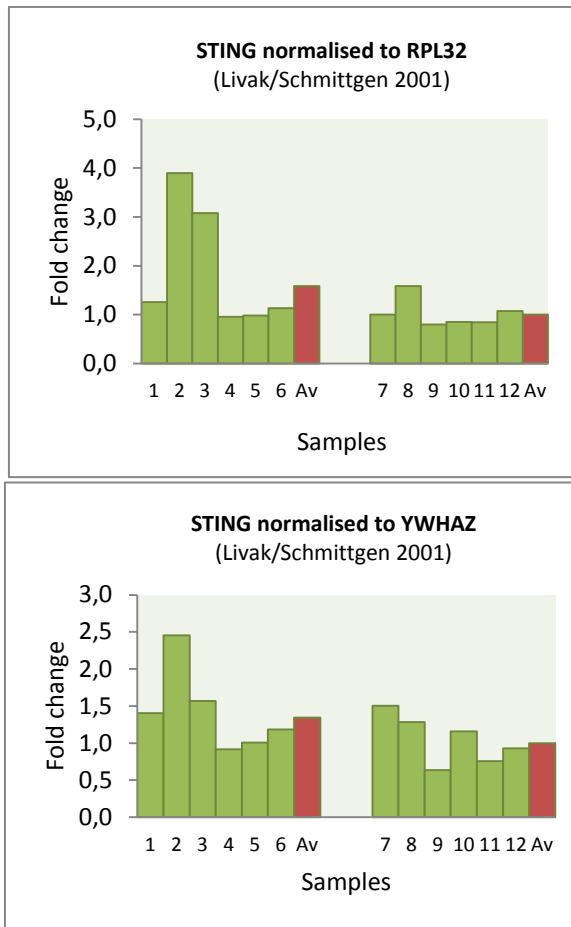


Figure 6: STING expression in samples (1-6, 7-12) normalised to RPL32 or YWHAZ (Livak/Schmittgen 2001)

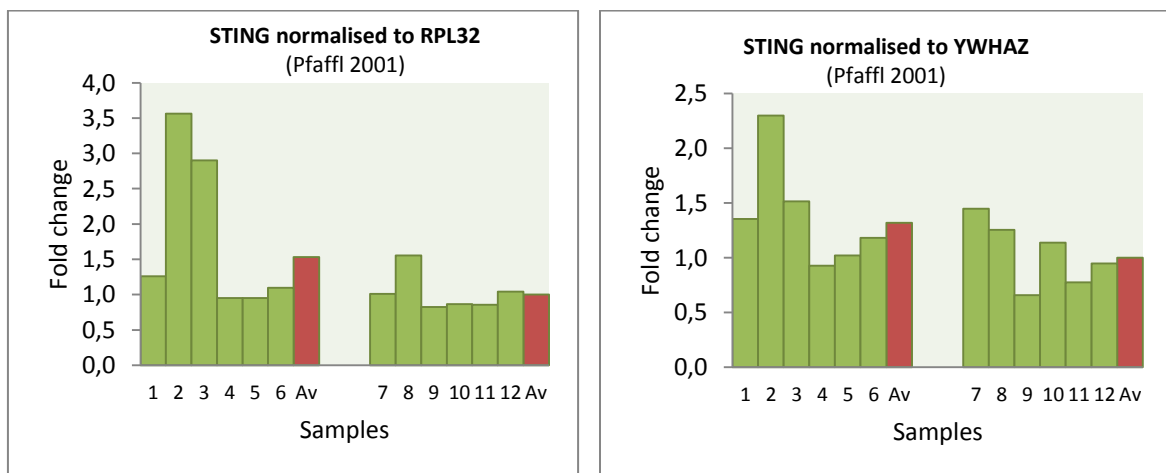


Figure 7: STING expression in samples (1-6, 7-12) normalised to RPL32 or YWHAZ (Pfaffl 2001)

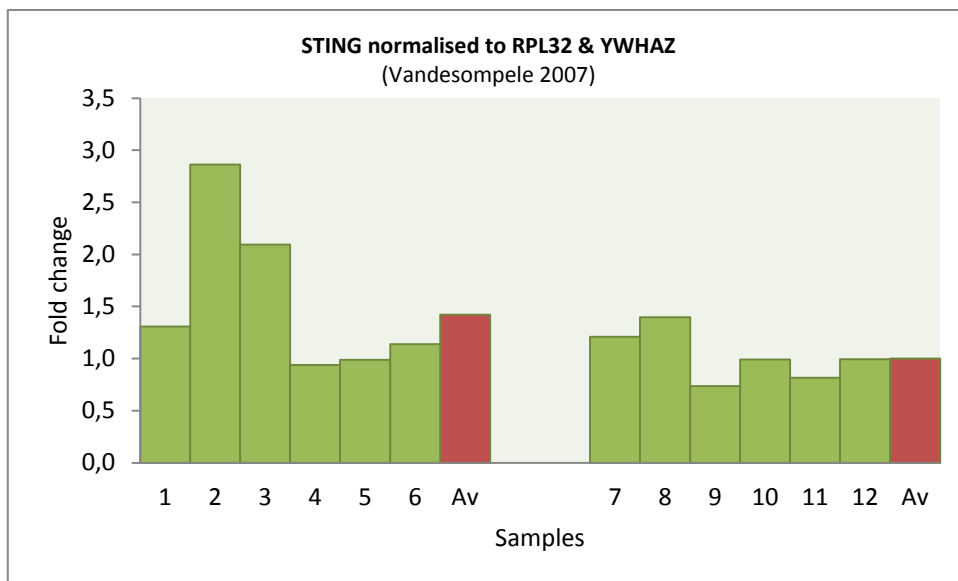


Figure 8: STING expression in samples (1-6 and 7-12) normalised to RPL32 & YWHAZ (Vandesompele 2007)

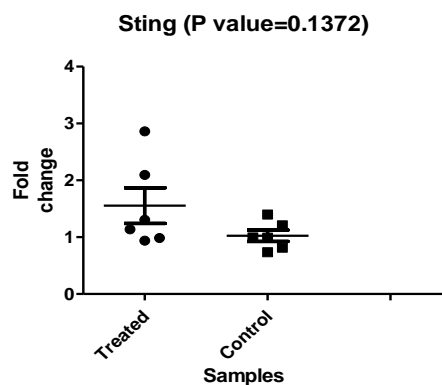


Figure 9: Grouped Analysis of STING on samples (A1-A6, K1-K6)

OPN:

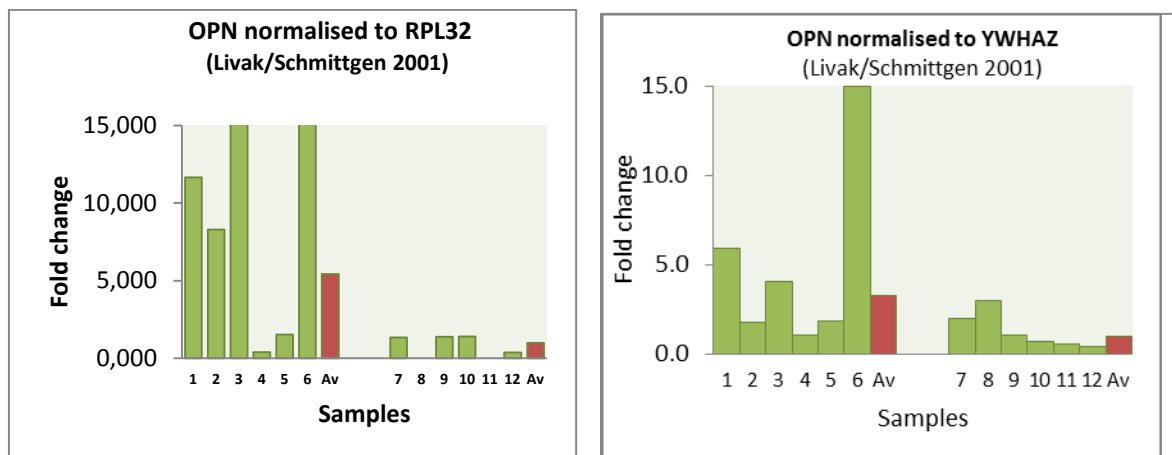


Figure 10: OPN expression on samples (A1-A6, K1-K6) against RPL32 & YWHAZ (Livak/Schmittgen 2001)

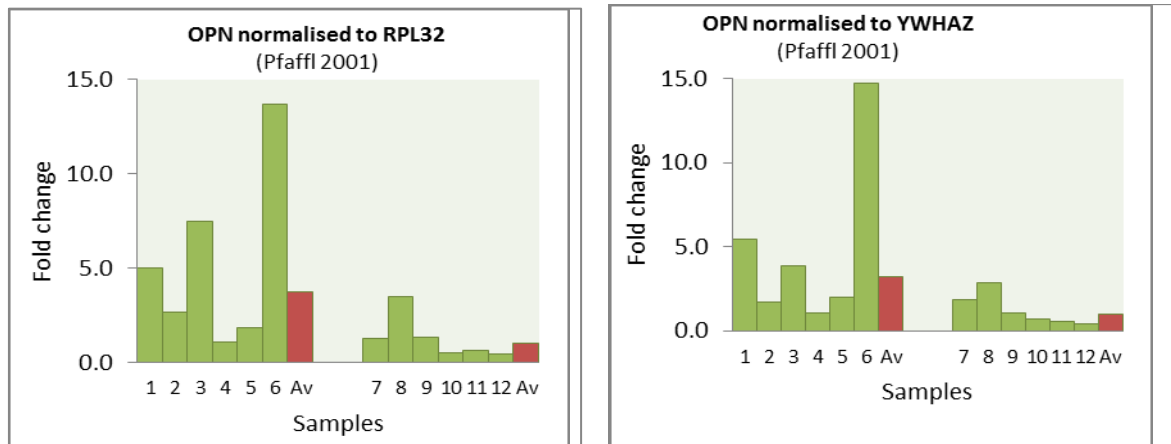


Figure 11: OPN expression on samples (A1-A6, K1-K6) against RPL32 & YWHAZ (Pfaffl 2001)

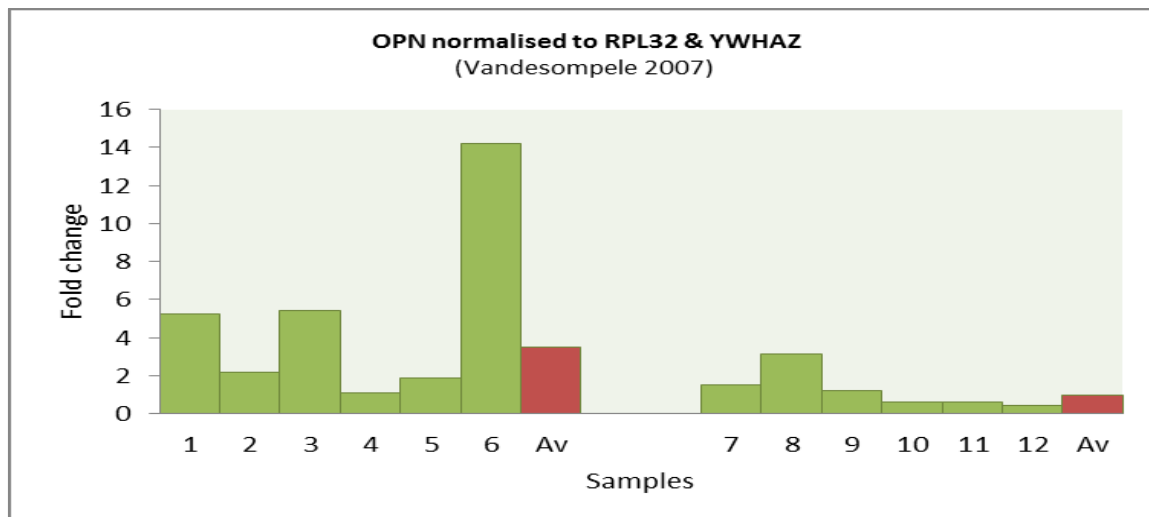


Figure 12: OPN expression on samples (A1-A6, K1-K6) against RPL32 & YWHAZ (Vandesompele 2007)

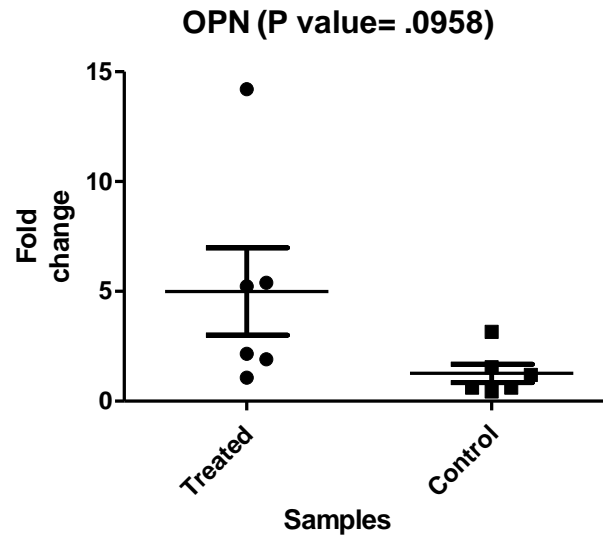


Figure 13: Grouped Analysis of OPN on samples (A1-A6, K1-K6)

IFN-β:

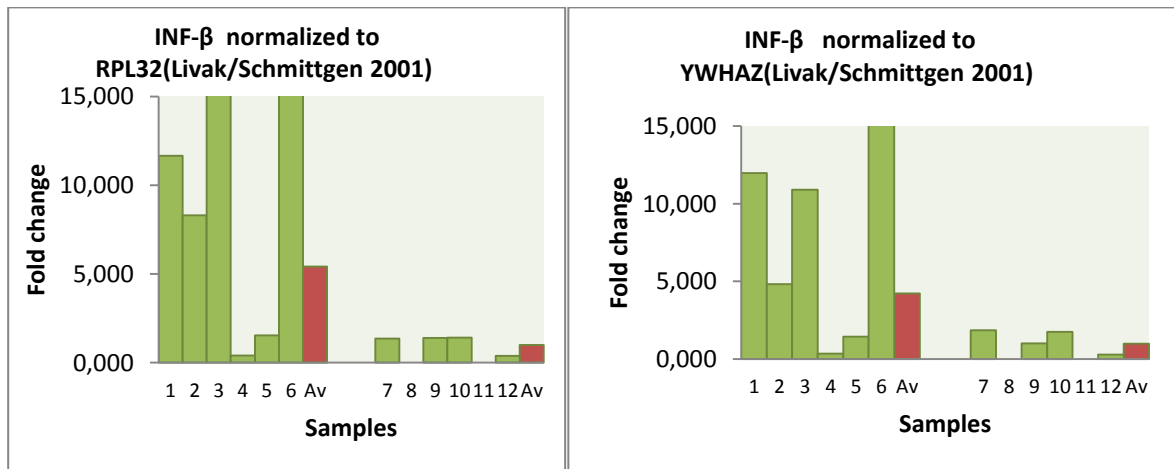


Figure 14: IFN-β expression on samples (A1-A6, K1-K6) against RPL32 & YWHAZ (Livak/Schmittgen 2001)

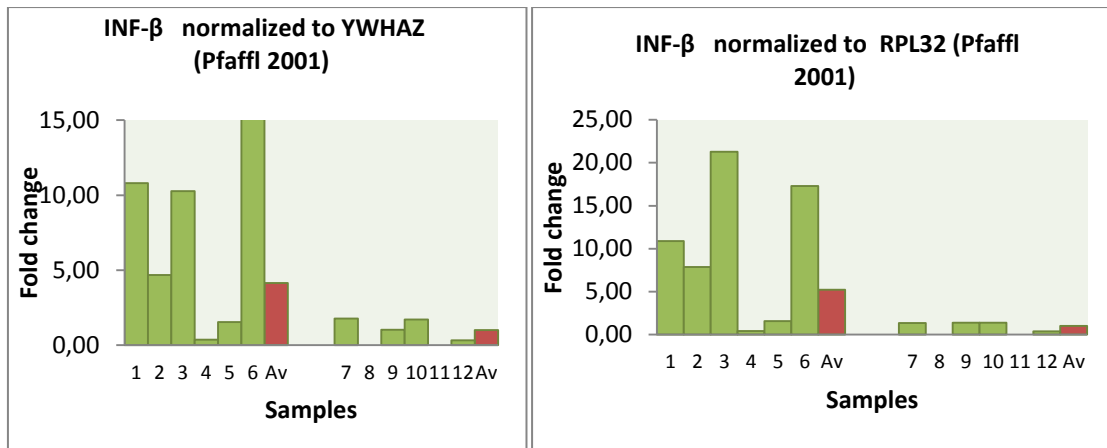


Figure 15: IFN-β expression on samples (A1-A6, K1-K6) against RPL32 & YWHAZ (Pfaffl 2001)

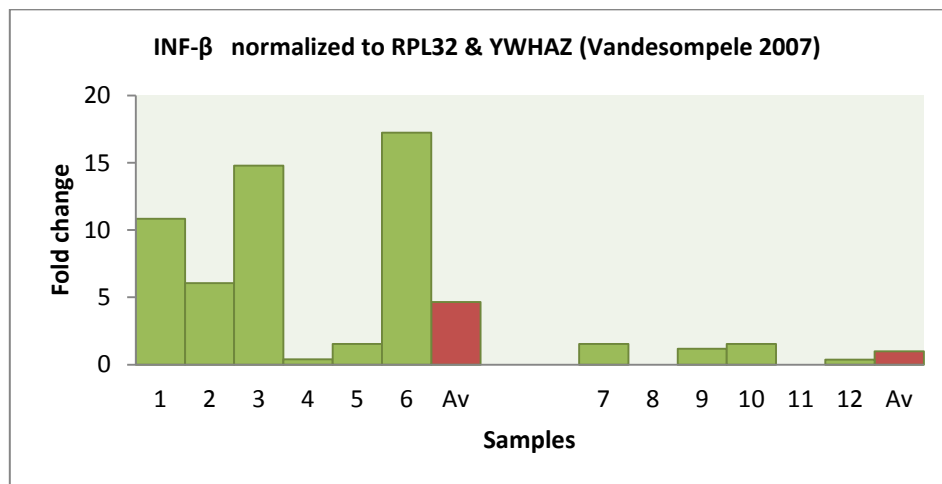


Figure 16: IFN-β expression on samples (A1-A6, K1-K6) against RPL32 & YWHAZ (Vandesompele 2007)

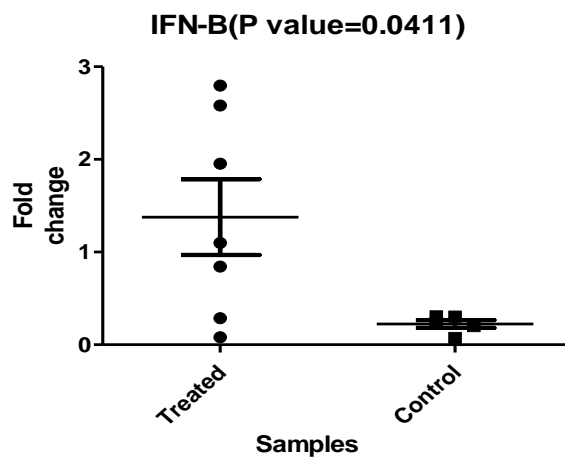


Figure 17: Grouped Analysis of IFN-β on samples (A1-A6, K1-K6)

The relative STING expression study carried out in animals A1-A6, K1-K6 showed elevated expression of STING in A1 and A2 when compared to the control group K1-K6. The expression of A1 and A2 was significantly different ($p < 0.05$) when compared to the control pigs (K1-K6). The relative gene expression study on OPN showed elevated expression in A1, A3 and A6 when compared to the control group K1-K6. A6 showed more than threefold increase in expression of OPN when compared to the other control group (K1-K6). The relative gene expression study on IFN- β showed elevated expression in A1, A2, A3 and A6 in comparison to the control group K1-K6.

4. CONCLUSION

The gene expression studies carried out using qPCR clearly suggested that there is an elevated expression of OPN, STING and IFN- β in four Matrix-M treated pigs compared to the six saline treated ones. This suggested that the upregulation of OPN and STING might contribute to the activation of Type I Interferons. In the experiment carried out, we clearly see that the Pigs A4 and A5 did not show elevated expression of STING or OPN or IFN- β . This evidently suggested that not all the pigs injected with Matrix -M responded to the treatment. Also the pigs that responded to Matrix-M did not show the same level of response. This inconsistency in treatment response makes it hard to carry out grouped comparison and, the expression between the two groups was only significantly different with respect to IFN- β (IFN- β $p = 0.0411$) and not significantly different ($p > 0.05$) for STING and OPN (STING $p = 0.1372$, OPN $p = 0.0958$). These differences are however in line with the high variation in type 1 interferon response among pigs (Liu et al., 2014).

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