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Research paper

RNA-seq analysis of viral gene expression in the skin of Marek's disease virus infected chickens

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ABSTRACT

Marek's disease virus (MDV), a highly cell-associated oncogenic avian α-herpesvirus, is the causative agent of malignant transformation of T cells in domestic chickens. The latently infected $CD4^+CD8^-$ T cells carry the virus through the blood stream and establish lymphomas in the skin, visceral organs and peripheral nerves. The feather follicle epithelium (FFE) is the only anatomical site where fully infectious enveloped virions are produced and eventually disseminated into the environment to infect contact birds. Therefore, skin and FFE play a critical role as being the common source of re-infection of birds sharing the same habitat. The molecular mechanism involved in the replication and assembly of MDV in the FFE leading to the production and release of cell-free infectious virus particles is unknown and to date no viral or host gene has been implicated in the process. To examine alterations in the expression pattern of viral genes, we performed RNA-seq on the skin samples of Marek's disease virus-infected susceptible chickens at 10, 20, and 30 days post infection. For comparative analysis of the expression patterns of viral genes between the skin and spleen of the MD-susceptible and resistant lines, Real-Time RT-PCR was employed. In total, RNA-seq based analysis identified 42 viral genes that were differentially expressed in the skin of infected birds. Majority of the identified genes are involved in DNA replication, capsid, tegument, and envelop formation. Comparative analysis between the skin and spleen of MD-susceptible and resistant chicken lines, revealed significantly higher expression of the genes in the skin of either lines than the spleen. Furthermore, much higher expression of the genes was observed in the skin of the susceptible line than the resistant line.

1. Introduction

Marek's disease virus (MDV) is a highly cell-associated lymphotropic α-herpesvirus and causative agent of a contagious lymphoproliferative and neuropathic disease commonly known as Marek's disease (MD) (Churchill and Biggs, 1967; Nazerian et al., 1968). MDV induces an initial lytic infection in B cells followed by a latent infection in activated $CD4^+$ T cells that persists up to three weeks before reactivation and transformation phase of infection (Calnek, 2001; Calnek et al., 1970; Churchill and Biggs, 1967). The latently infected T lymphocytes are believed to be the means of virus transfer to the skin and feather follicle epithelium (FFE), the only anatomical site where infectious fully enveloped cell-free virions are assembled and released into the environment to infect contact birds (Calnek et al., 1970; Witter et al., 1972). Immunofluorescence and transmission electron microscopic studies in addition to skin lesions induced by MDV provided earlier evidence that skin and FFE might be involved in dissemination of the virus into the environment (Couteaudier and Denesvre, 2014). It was subsequently shown that intraabdominal inoculation of dust, dander, and feather debris collected from isolators housing MDV-infected birds or homogenates from skin of infected birds with feather tips induce MD in naïve healthy birds (Calnek and Adldinger, 1971; Nazerian and Witter, 1970). The affected cells of the skin are the stratified squamous epithelium that commonly detach or slough off with molted feathers, spreading the virus into the environment. MDV spreads horizontally to contact birds via dander and dust in poultry houses (Calnek and Adldinger, 1971; Witter and Kreager, 2004). MDV antigens and the release of cell-free virus particles from the skin and FFE can be detected through the life of the infected chickens (Calnek et al., 1970). It is postulated that virus interaction with the skin is the major reason for

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MD persistence in poultry houses and the drive for the evolution toward more virulent pathotypes (Nair, 2005; Witter, 1997). The molecular mechanism involved in the production of cell-free enveloped virions, however, is not known and MDV genes associated with replication, assembly and egress in the FFE have not been identified.

MDV is classified as an alpha-herpesvirus based on its genomic structure and sequence homology (Buckmaster et al., 1988; Cebrian et al., 1982). The prototype of α-herpesviruses, herpes simplex virus type1 (HSV-1), consists of 4 morphologically distinct structural components that include a DNA core, an icosahedral capsid, surrounded by a proteinaceous layer known as tegument, and an envelope that consists of viral proteins, host-cell derived lipids and cell membrane (Mettenleiter, 2002). Sequence analysis and gene arrangement within the family of herpesviridae, have identified close to 40 conserved genes that play essential roles in virus replication and assembly (Mettenleiter, 2002, 2006; Mettenleiter et al., 2009; Pellett et al., 1985). Of HSV-1 genes, UL18, UL19, UL35, UL38, UL6, and UL25 are among the genes that have been identified as components of nucleocapsids (Mettenleiter, 2006). The majority of 23 virally encoded proteins involved in the formation of tegument are conserved among all three herpesvirus subfamilies (alpha, beta, and gamma herpesviruses) with UL7, UL11, UL16, UL21, UL36, UL37, and UL51 being unique to alpha-herpesviruses (Kelly et al., 2009; Loret et al., 2008; Newcomb et al., 2012).

In addition to 16 different virally encoded genes that are implicated in secondary envelopment, cellular proteins including actin, hsp70, hsp90, moisin, b-tubullin, and annexin have also been detected (Maxwell and Frappier, 2007). Whether the virally encoded proteins found in enveloped virus particles (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gM, gN, UL20, UL43, UL45, UL56, and US9) are transported to the site of the secondary envelopment independently or as preformed subcomplex structure, is not known (Owen et al., 2015). Some of these proteins contain trafficking motifs that mediate endocytosis and localization to the site of the secondary envelopment compartments. Some, on the other hand, lack such motifs and perhaps rely on other viral proteins as chaperons for localization and final assembly in the virus envelopment (Beitia Ortiz de Zarate et al., 2007; Van Minnebruggen et al., 2004).

Homologues of HSV-1 tegument proteins (UL46, UL47, UL48, and UL49) involved in tegument formation have also been identified in MDV (Yanagida et al., 1993). However, only a bicistronic mRNA corresponding to UL49-UL48 genes has been detected in both in vivo and in vitro expression systems that direct the synthesis of VP16 and VP22 (UL48 and UL49, respectively) (Dorange et al., 2000). Mutant MDV constructs with UL46, UL47, UL48, and UL49 deletion individually or in combination has revealed that UL46, UL47, and UL49 are dispensable for MDV growth in chicken embryonic skin and quail muscle cells. MDV Mutant virus with UL49 deletion, however, did not generate plaques in cell culture and inhibited cell-to-cell spread (Dorange et al., 2002). It was shown that co-expression of MDV genes homologues to HSV1 UL18, UL19, UL26.5, and UL38 in insect cells resulted in capsid formation with a large core (Kut and Rasschaert, 2004). Further deletion studies have provided evidence that UL13 and UL44 (encoding serine/threonine and glycoprotein gC, receptively) are essential for horizontal transmission of virus from bird to bird, suggesting involvement of the proteins in envelop formation and maturation of virions (Jarosinski et al., 2007; Jarosinski and Osterrieder, 2010; Jarosinski and Vautherot, 2015).

The goal of this study was to provide insight into possible differential expression of MDV genes in the skin and FFE that provide a safe haven for production of cell-free, enveloped, and infectious virions. To this end, we performed gene expression profiling on the skin samples of MD-susceptible chickens during lytic, latency and reactivation phases of viral infection via RNAseq. For comparative analysis of gene expression patterns between the skin and spleen tissues of MD-susceptible and resistant chickens, qPCR was employed.

2. Material and methods

2.1. Experimental chickens

The specific-pathogen-free chickens in this study were from the highly inbred MD-susceptible Line 7_2 and resistant Line 6_3 (Bacon et al., 2000). These birds were from unvaccinated breeder hens and therefore, carried no maternal antibodies to MDV or herpesvirus of turkeys. All animal experiments were approved and carried out in accordance to the guidelines set forth by the Avian Disease and Oncology Laboratory Institutional Animal Care and Use Committee and the Guidelines for Care and Use of Laboratory Animals published by Institute for Laboratory Animal Research (ILAR Guide) in 1996 (http://www.nap.edu/openbook.php?record_id=5140).

2.2. Virus

A Bacterial Artificial Chromosome (BAC)-cloned very virulent (vv) strain of MDV, rMd5, was used in this experiment (Niikura et al., 2011).

2.3. RNA isolation

The RNA samples were isolated from the homogenized spleen and skin tissues of four birds from each group at 10, 20, and 30 days post infection (dpi) using Tri Reagent RT according to the manufacturer's instructions (Molecular Research Center, Cincinnati, OH). The RNA samples were processed and analyzed individually and were not pooled (four biological replications). For skin tissues, the feathers were clipped at the base with tips remaining in the skin.

2.4. RNA sequencing

The RNAseq for skin samples of Line 7_2 was conducted at the Research Technology and Support Facility RTSF of Michigan State University in East Lansing, Michigan using Illumina TruSeq Stranded mRNA kit and reagents following the manufacturer's protocols. In brief, the chemically fragmented polyA mRNA isolated from 1 μg total RNA was reverse transcribed to form double stranded cDNA. To create the final libraries, the cDNA samples were end repaired, A-tailed, adapter ligated and amplified. After quantification of all the libraries on a Qubit Fluorometer (Life Technologies, Carlsbad, CA), Agilent BioAnalyzer was used to determine final size distribution and purity of the library. The final concentration of the libraries was determined by QPCR using the KAPA Illumina Library Quantification Kit (KAPA Biosystems, Wilmington, Massachusetts), appropriately diluted and loaded onto the flow cell for sequencing on the Illumina HiSeq2500 following the manufacturers protocols.

2.5. Real-time PCR

Four micrograms of total RNA isolated from the skin and spleen of each of the individual chickens (Both lines 6_3 and 7_2) were used for cDNA synthesis using SuperScript III First-Strand Synthesis System for Reverse Transcriptase PCR (Invitrogen, Carlsbad, CA) following the manufacturer's directions. For each RNA samples, three replicates of Real-Time PCR were performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) following the manufacturer's directions and with 100 ng of cDNA and 0.5μM each of forward and reverse primers per reaction. The amplification program included: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 57 °C for 1 min. The Primer-BLAST (NIH, Bethesda, MD) was used to design primers for MDV genes (Table 1). The designed primers were synthesized by Eurofins MWG Operon LLC (Huntsville, AL). The expression patterns of MDV genes identified in line 7_2 by RNA-seq, were also analyzed by Real-Time PCR in the skin samples of both MD-susceptible and resistant lines. To compare the gene expression pattern between

Table 1
Primer sequences used in Real-Time Polymerase Chain Reaction.

MDV Gene ID	Forward Primers	Reverse Primers
MDV005	5'-CAGGATTCCTTGTATTCGGGC-3'	5'-TAGGGGAGAAGAAACATGGGG-3'
MDV010	5'-GCTCGAAACATTAGGGCCAAT-3'	5'-AAGATCGGTAGGAATGCAGGT-3'
MDV013	5'-GTCAAGAGAGGAGACCCTGGA-3'	5'-GGGAGATGGATGGCTGTATTT-3'
MDV014	5'-TTCGGTAGGATGGCAGTTCTT-3'	5'-TGGATAAGGGAGATGGATGGC-3'
MDV015	5'-ATTCTTCTGGTTCGTGAGTGC-3'	5'-CTGCGTAATATGCTTGCTGGA-3'
MDV015.5	5'-AGATTATGGCTCCCCCTGAA-3'	5'-ATGGGTGGACAGCGAAATAA-3'
MDV016	5'-GGCAAATCCGACTGACACTTT-3'	5'-GGTGATGGATAATTGACGGGG-3'
MDV019	5'-CCCTGCGTTCAAAGGTTTCAT-3'	5'-GAGATGAGCAAACAAAGGGCA-3'
MDV020	5'-TATGTAGATACGGACGGCCTG-3'	5'-GTCCTGTTGTCCATTCATCGG-3'
MDV023	5'-GGCCAAGCAGTGTCGTATTTA-3'	5'-GTTGCATGAGAGGATCGGAAC-3'
MDV024	5'-AGCATCATCGGTAAAGTGGAG-3'	5'-AGATTGGCTGACACAGAGTGC-3'
MDV025	5'-AATCCCCGCCATCAAAACTTT-3'	5'-ACGAACAATGAAAAGCACCGA-3'
MDV028	5'-TATGCCATTATCCCTGATGTG-3'	5'-GCCAGTAAACTCTCTTCCATT-3'
MDV029	5'-TATCTTGTTCCTCGCAAATGG-3'	5'-GGTGTCGTCGGTAAAAACTCA-3'
MDV030	5'-CCTACGCTACTTACATTGGGG-3'	5'-AAAGTGCCTCATAGACGGGAT-3'
MDV031	5'-TGCCGCCCACTCATTCATATA-3'	5'-GCACGGAAGCTCAATCTCTAC-3'
MDV034	5'-AGTTTTGCCCATTTCTGGACT-3'	5'-GTGTGTCTAAGGTTTCCGTTT-3'
MDV037	5'-AGAATCAATGTTCTGCGTGCA-3'	5'-GTTGGGAATGTGCGTTTCAAG-3'
MDV038	5'-AGTGTGTATCAGTCCAGCACA-3'	5'-GGGCGAGAAGATTGAGTGTTC-3'
MDV039	5'-TACCCATAACTCTCTGCTTCC-3'	5'-ACTGGTCTCTTTTCCTTTGTC-3'
MDV040	5'-ATGTATTAAACGCGAGGCAGA-3'	5'-ATGAGCCAGGGATTTGGATAG-3'
MDV044	5'-CACCGCTTATCAAGACAACGT-3'	5'-CTCTGTCGTTTCGTCCCAAAC-3'
MDV047	5'-CCGAACATAAATAGTCGCCGT-3'	5'-TGCTCCACTAAACTGAAACGC-3'
MDV048	5'-TGAAGTACGCCGACATAACGA-3'	5'-GTTGGGAATGTGCGTTTCAAG-3'
MDV049	5'-GACGGATTAGAACAGAATTGC-3'	5'-AGTAGTTACATTTCCCGTGAG-3'
MDV053	5'-ATTCGACAATGACTCACTGGC-3'	5'-GATGCGGAGAAGAAAATGCCT-3'
MDV056	5'-GAGTCTGTGGCGTTTTAGTGG-3'	5'-TGCGGAAAATAAAGTTGGCCC-3'
MDV059	5'-AAGTGGATGAGCACTGCATTG-3'	5'-GAGAGAAACCAACGCGACAAT-3'
MDV062	5'-ATTCCTACCACCAATCACTGC-3'	5'-CCTCGGTAAGCATTGAAACGT-3'
MDV067	5'-CGATGGGCTGCCGTATTAAC-3'	5'-CTTCAGCACTCCCTAATGCG-3'
MDV068	5'-CAAGGCGACGATGACTCAAAT-3'	5'-GCCGTTTAGTTTGGGAAATGC-3'
MDV071	5'-CGCGTCAGGAGTTTTGAGTAC-3'	5'-TCCGTTGAAGTGAGAGGGAAA-3'
MDV072.5	5'-ACCAATCGAGGAAGAACCTG-3'	5'-GGGATAGGGAATCGTATGAAG-3'
MDV076	5'-CACTGTGCGATTCTAAGTGCT-3'	5'-TCTGCCTATGTGCCTGTGTAT-3'
MDV088	5'-CCCACGCCAAAGTCTACATC-3'	5'-CCGACAAATCGTTCATTCCT-3'
MDV089	5'-AAACCACCATCGTCTCGTTTG-3'	5'-GAGGAGCACGTTCAATACACC-3'
MDV090	5'-AACTGGACCACAATGCTTTCC-3'	5'-GCACACAGGTAGAGCAAGAAC-3'
MDV093	5'-TCCCACAGACCGCAAATAAAG-3'	5'-ACTTTTAGGGTCCTCTGCCTG-3'
MDV095	5'-CCAGACTCGGACCAGTTCTT-3'	5'-TACCACAGCGTCTCTACAGC-3'
MDV096	5'-ACGACGATCAAAGTAGCTGGA-3'	5'-TTTCGTTGCAGTGATTGGGAA-3'
R-LORF4.2	5'-TTTGGGTAATTGGTCTGCGC-3'	5'-AGAAAATTCCCACGACCCCT-3'
VTR	5'-CTCCGCTGTGTCTAACCCTAA-3'	CCCTTTCGGTCCTTTCTCCC-3'

skin and spleen tissues of birds from each line, Real-Time PCR was performed on RNA samples isolated from the spleen tissues of infected birds of both susceptible and resistant lines.

Relative quantification of MDV genes was determined using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The samples from the earliest time point (10dpi) were used as baseline and the expression levels of the viral genes at 20 and 30dpi were compared to those of 10dpi. The expression of each gene was normalized to the expression level of the housekeeping gene, *β*-actin. Statistical analysis was performed with the aid of GraphPad software (GraphPad, La Jolla, CA) using an unpaired *t*-test for determining fold changes between samples and corresponding P values.

2.6. Bioinformatics analysis

The Illumina reads were trimmed by CLC Genomics Workbench version 8.0 (CLC bio, Cambridge, MA, USA) and trim sequences algorithm (Parameters: Minimum base pairs 75, Quality limit 0.05, Ambiguity limit 2, Adapter screen yes). Trimmed reads were then used in a batch RNA-seq experiment using CLC Genomics Workbench version 8.0.2 (CLC bio, Cambridge, MA, USA) to the MDV reference genome, (NC_002229.3), (Parameters: Similarity 0.8; Length fraction 0.8; Insertion cost 3; Deletion cost 3; Mismatch cost 2). The generated RNA-seq mapping resulted in per library TPM and read counts for the genes in the reference genome. For TPM calculation, the read counts were divided by the length of each gene in kilobases. This gave the reads per kilobase (RPK). Then all the RPK values were counted up in a sample and divided by 1,000,000. This gave per million scaling factors. Finally, the RPK values were divided by the per million scaling factors to obtain TPM (Transcript per million).

2.7. Genomic DNA isolation

Genomic DNA was isolated using the Gentra Puregene kit (Qiagen, Hilden, Germany) following manufacturer's directions. Briefly, sections of skin tissue stored in RNAlater (Thermo Fisher, Grand Island, NY) were washed in PBS and blot-dried. Approximately 100 mg of blot-dried skin tissue was homogenized and mixed with 400 µl of cell lysis solution (Gentra Puregene kit, Qiagen, Hilden, Germany) with Proteinase K (100 µg/µl, final concentration). Tissue lysates were incubated overnight in a heat block shaker at 60 °C and 900 rpm. To exclude RNA contamination, the tissue lysates were incubated with RNase A for 30 min at 37 °C followed by the addition of protein precipitation solution and centrifugation. The supernatants were transferred to a clean microcentrifuge tube and 400 µL isopropanol was added. Samples were inverted and centrifuged again, and the DNA pellet was washed in 70% ethanol and air-dried. DNA pellets were rehydrated in clean water. To remove lipid and fat contamination, all DNA solutions were mixed with chloroform:isoamyl alcohol, centrifuged, and the aqueous layer was removed for further analysis. DNA concentrations of samples were

quantified using a NanoDrop 8000 (Thermo Scient, Grand Island, NY).

2.8. MDV genome copy number assay

The quantitative Real-Time PCR (qPCR) for MDV genome copy number analysis was according to the previously described protocol (Gimeno et al., 2008). Analysis of genomic DNA from each skin sample was performed in triplicate on a single 96 well plate. qPCR assays were performed with a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Primers to MDV gB and chicken GAPDH were each used at 0.5μM to amplify their respective genes. Probes to MDV gB and chicken GAPDH were used at 0.2 μM. MDV loads were shown as the copy number of MDV gB divided by the copy number of chicken's GAPDH. Statistical analysis was performed with the aid of GraphPad software (GraphPad, La Jolla, CA) using an unpaired *t*-test.

2.9. Experimental design

Thirty-four one-day-old chicks from each of the Line 7_2 (MD-susceptible) and 6_3 (MD-resistant) were randomly distributed into two groups of 17 each in separate isolators. Birds from one group were inoculated intraperitoneally with 2000 pfu of rMd5 at 12 days post-hatch. The second group served as the un-inoculated negative control. Seventeen birds were designated per group to ensure the desired replicate number (4) per sample interval, as some mortality is expected post-hatch and/or from MDV-induced complications. At 10, 20, and 30dpi, four birds from each group were euthanized by CO_2 inhalation and necropsied for tissue collection. Skin and spleen tissues were collected and stored in RNAlater (Thermo Fisher Scientific, Grans Island, NY) to prevent RNA degradation. Skin samples were from the dorsal cervical and capital tracts of individual birds. Samples were kept at −20 °C until used for RNA and DNA isolation and gene expression profiling. This study also included host gene expression profiling that has previously been published (Heidari and Delekta, 2017).

3. Results

3.1. MDV genome load in the skin of MDV-infected chickens

To determine the relative viral genome copy number in the skin of infected chickens of MD susceptible (7_2), we performed qPCR on DNA samples isolated from the skin samples at 10, 20, and 30dpi using primers specific to MDV gB and host GAPDH. A considerable number of virions were detected in the skin tissues of infected chickens at each time point post-infection. No viral genome was observed in the tissue samples of uninfected control birds (Fig. 1). This figure is from our previous publication (Heidari and Delekta, 2017) and is included in this manuscript with permission from the Publisher.

3.2. Summary of the RNA-seq data set

We performed RNA-seq analysis to identify differentially regulated viral genes that may play a role in production and assembly of cell-free enveloped virus particles in the skin and FFE. We divided the birds into two groups and infected half with rMd5 at 2000 pfu and then collected skin samples from four biological replicates per treatment group at 10, 20, and 30dpi. We then isolated RNA from each individual skin sample for independent sequencing. We used high-throughput sequencing to generate 19,435,174 to 66,996,560 raw reads per sample, including host genes that has been reported (Heidari and Delekta, 2017). The total reads from infected samples were mapped to the MDV reference genome using CLC Genomics Workbench version 8.0 software. Of these mapped reads, we identified 42 viral genes that are involved in DNA replication, capsid, tegument, and envelop formation (Table 2). The orthologues of these identified MDV genes in human HSV-1 and varicella-zoster virus (VZV) including their known biological functions, as well as TPM (Transcripts per million) of the gene Reads are also reported.

3.3. RT-PCR analysis of MDV genes in the skin and spleen tissues of infected birds

To verify the expression pattern of MDV genes identified in RNA-seq analysis in the skin of MD-susceptible line, we employed Real-Time PCR on the RNA samples isolated from the skin of infected birds at 10, 20, and 30dpi (Supplementary Fig. 1). For comparative analysis, we also tested the expression pattern of all 42 genes in the skin samples of the resistant line (Supplementary Fig. 2). To shed light on the differential expression levels of the MDV genes detected in RNA-seq analysis, we conducted Real-Time-PCR on the spleen tissues of both susceptible and resistant lines (Supplementary Figures 3 and 4, respectively). Comparative analysis of a few randomly selected MDV genes between the skin of the MD-susceptible and resistant lines highlights the significantly up regulated genes in the skin samples of the susceptible birds than those of the resistant ones (Fig. 2). Same pattern of MDV gene expression was detected when the spleen tissues of MD-susceptible line was compared with those of the resistant line (Fig. 3). Statistical analysis was conducted to determine fold changes and P values of RT-PCR based MDV gene expression levels in the skin and spleen samples of both the susceptible (7_2) and the resistant line (6_3) at all three time-points (Table 3 and 4, respectively).

4. Discussion

The latently infected $CD4^+CD8^-$ T cells are believed to be the means of virus transfer to the skin and FFE cells where cell-free enveloped infectious virions are produced and disseminated into the environment (Calnek et al., 1970; Witter et al., 1972). In addition to being the common source of re-infection of chickens in poultry houses, skin and FFE cells play a critical role in the evolution of MDV toward higher virulence (Baigent et al., 2005). Host and viral gene expression pattern in the FFE cells are undoubtedly different from other anatomical sites and are likely playing an essential role in creating a safe haven for virus replication and evasion of the immune responses. The molecular mechanism underlining the replication and assembly of virus particles in the FFE cells and production of cell-free enveloped virions are unknown. To provide insights into the expression pattern of MDV genes in the skin and FFE cells of infected birds and shed light on the differential expression of the viral genes between skin and spleen, we performed gene expression profiling in the skin and spleen tissues of MDV-infected chickens at three different time points post infection employing RNA-seq and Real-Time PCR.

The genome copy number of rMd5 that represents replication rate and assembly of the virus, showed a typical pattern of production for a wild type virus within the skin and FFE cells. The slower rate of replication in the early days of infection followed by a massive number of virion production between 3- and 4-weeks post infection. By 30dpi, the virus replication typically slows down but continues throughout the life of the infected chickens (Fig. 1).

Using RNA-seq analysis, 42 viral genes were detected with at least 25 exhibiting higher read counts at different time points post inoculation (Table 2). Majority of the detected genes are associated with DNA replication, capsid, tegument, and envelop formation. UL18, UL19, and UL26.5 are nucleocapsid associated proteins with high read counts at 10, 20, and 30dpi. UL35, another capsid related protein, had low read counts and consequently, TPM values were non-significant. UL25, a gene involved in DNA packaging, had some of the highest reads among all the identified genes. Of the genes involved in tegument formation, UL7 and UL36 exhibited high transcriptional activities at all three time-points post-infection. Glycoprotein H (gH), gB, gK, gI, and gE with high read counts, are all found in enveloped virus particles (Owen et al., 2015). LORF10 that is also speculated to be involved in envelop

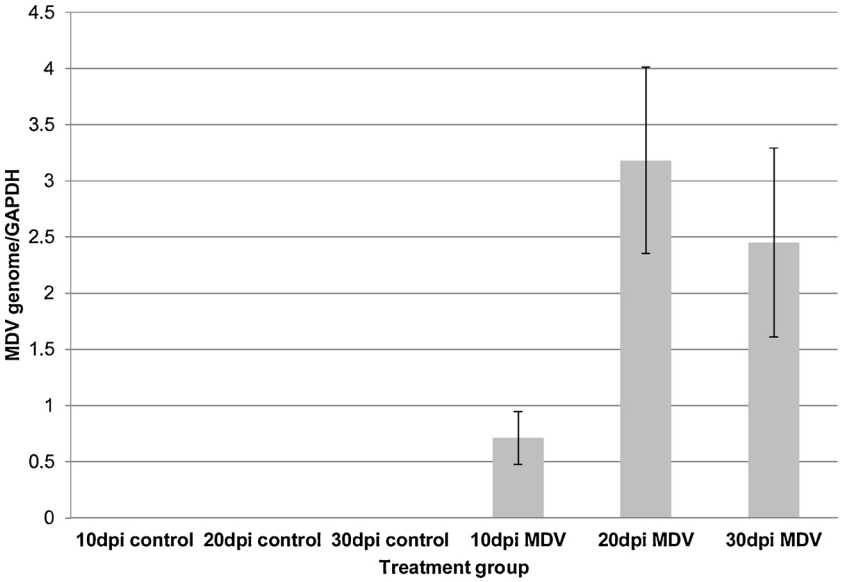

Fig. 1. shows the genome copy number of rMd5 in the skin of infected birds at 10, 20, and 30dpi. Quantitative real- time polymerase chain reaction analysis for MDV genome copy number was performed on DNA isolated from skin tissue samples of infected and uninfected birds. Probes corresponding to MDV gB and host GAPDH were used to quantify each gene. Each result is representative of four chickens that were individually evaluated. Viral loads were conveyed as the copy number of MDV gB divided by that of the host GAPDH. Statistical analysis was performed using an unpaired *t*-test and all data points had a p-value < 0.05. MDV, Marek's disease virus.

Table 2
RNA-seq based MDV genes identified in the skin of infected chickens.

MDV Gene ID	MDV ORF	HSV1 Orthologues	VZV Orthologues	Gene Function	TPM Skin of MDV-infected chicken line 7-2		
					10 dpi	20 dpi	30 dpi
MDV005	R-LORF7			Oncoprotein MEQ (jun/fos homolog)	2252.89	4649.42	10688.78
MDV010	LORF2			Phospholipase (exon 1)	0.00*	0.00	1597.22
MDV013	UL1	UL1	ORF60	Glycoprotein L (gL), complexed with gH	0.00	0.00	0.00
MDV014	UL2	UL2	ORF59	Uracil DNA glycosylase	0.00	0.00	0.00
MDV015	UL3	UL3	ORF58	Nuclear phosphoprotein	0.00	1443.14	7249.01
MDV015.5	UL3.5	UL3.5		Tegument protein; virus egress	0.00	0.00	0.00
MDV016	UL4	UL4	ORF56	Late nuclear protein	1591.59	1204.84	1234.31
MDV019	UL7	UL7	ORF53	Tegument protein	871.15	203.10	1150.71
MDV020	UL8	UL8	ORF52	DNA Helicase/Primase associated protein	0.00	0.00	562.46
MDV023	UL11	UL11	ORF49	Myristylated tegument protein	0.00	0.00	0.00
MDV024	UL12	UL12	ORF48	Deoxyribonuclease	297.77	556.10	6723.48
MDV025	UL13	UL13	ORF47	Serine/threonine proteine kinase (tegument)	0.00	0.00	0.00
MDV028	UL16	UL16	ORF44	Tegument protein	0.00	0.00	0.00
MDV029	UL17	UL17	ORF43	Tegument protein, DNA packaging	0.00	0.00	0.00
MDV030	UL18	UL18	ORF41	Nucleocapsid protein (VP23)	4237.33	3903.78	6556.65
MDV031	UL19	UL19	ORF40	Major capsid protein (V5)	1345.52	2036.84	2880.42
MDV034	UL22	UL22	ORF37	Envelop glycoprotein H (gH)	0.00	0.00	0.00
MDV037	UL25	UL25	ORF34	DNA packaging	16862.30	13737.57	9177.12
MDV038	UL26	UL26	ORF33	VP24 capsid maturational protease, scaffold protein	13182.18	16096.10	21268.82
MDV039	UL26.5	UL26.5	ORF33.5	Minor capsid scaffold protein	0.00	0.00	832.35
MDV040	UL27	UL27	ORF31	Glycoprotein B (gB)	4235.03	4102.81	4208.09
MDV044	UL31	UL31	ORF27	Nuclear phosphoprotein	0.00	0.00	0.00
MDV047	UL34	UL34	ORF24	Nuclear egress membrane protein	5761.99	3585.46	820.97
MDV048	UL35	UL35	ORF23	Small capsid protein	0.00	0.00	0.00
MDV049	UL36	UL36	ORF22	Large tegument protein	360.63	253.12	250.75
MDV053	UL40	UL40	ORF18	Ribonucleotide reductase small subunit	0.00	0.00	0.00
MDV056	UL43	UL43	ORF15	Membrane protein	0.00	0.00	0.00
MDV059	UL46	UL46	ORF12	VP11/VP12 tegument phosphoproteins	7693.93	15693.94	17581.45
MDV062	UL49	UL49	ORF9	VP22 phosphoprotein (tegument)	0.00	0.00	0.00
MDV067	UL53	UL53	ORF5	Glycoprotein K (gK)	4438.93	2694.49	4050.03
MDV068	UL54	UL54 (ICP27)	ORF4	ICP27	6275.76	7214.89	5910.30
MDV071	LORF10		ORF2	Myristylated tegument protein	1164.97	1542.88	523.87
MDV072.5	UL56			Membrane protein	0.00	0.00	0.00
MDV076	R-LORF7			MEQ (jun/fos homolog)	1757.63	3570.93	9964.34
MDV088	US1, ICP22	US1	ORF63	Regulatory protein, ICP22	0.00	0.00	0.00
MDV089	US10	US10	ORF64	Capsid/tegument associated phosphoprotein	0.00	0.00	695.82
MDV090	SOR3			SORF3 hypothetical protein	8117.09	11807.52	14502.76
MDV093	SORF4			SORF4 (hypothetical protein)	0.00	0.00	140.74
MDV095	US7	US7	ORF67	Glycoprotein I (gI)	5998.99	10746.51	12399.25
MDV096	US8	US8	ORF68	Glycoprotein E (gE)	0.00	1170.34	583.89
R-LORF4.2				Contains a potential transmembrane domain	564.79	693.97	520.43
VTR				Viral telomerase	32.16	677.04	7244.07

The original counts were low but above zero.
* The TPM of gene with low transcriptional counts (Read counts) becomes zero after normalization.

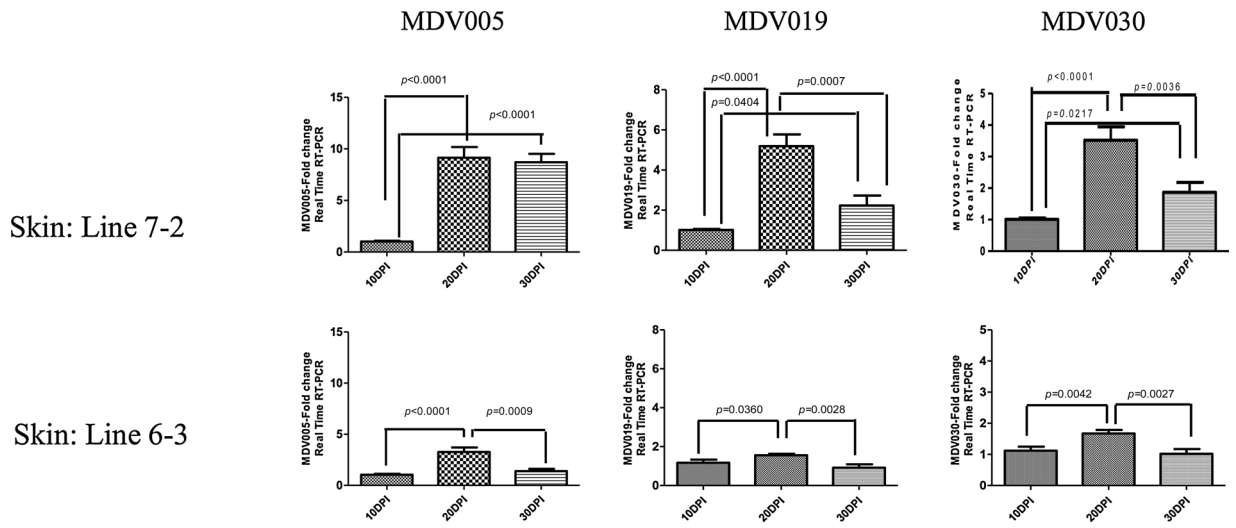

Fig. 2. displays comparative analysis of three randomly selected viral genes between the skin of the susceptible line 7_2 and the resistant line 6_3 at 10, 20, and 30dpi. Statistical analysis was performed using an unpaired *t*-test.

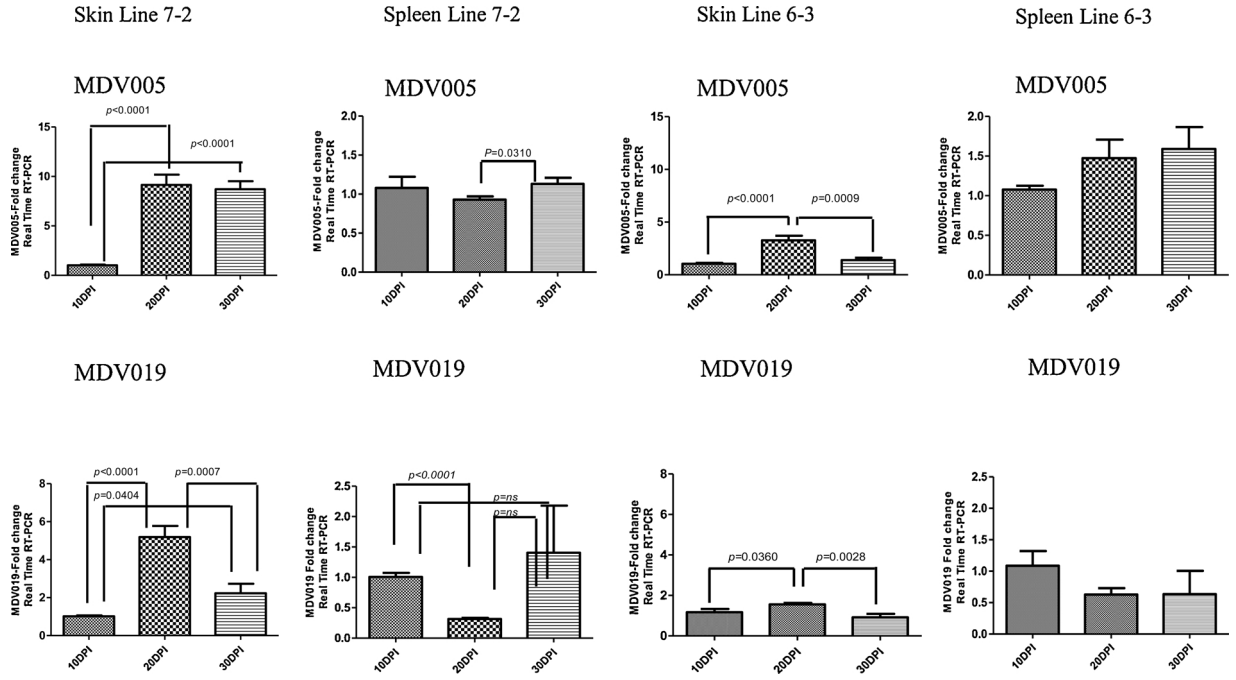

Fig. 3. depicts comparative analysis of two randomly selected viral genes, MDV005 and MDV 019, between the skin and spleen tissues of the susceptible line 7_2 and the resistant line 6_3 at 10, 20, and 30dpi, respectively. Statistical analysis was performed using an unpaired *t*-test.

formation, showed higher transcriptional activities in the skin of infected birds at all time-points post infection. Some of the genes that are known to be associated with capsid or tegument formation in HSV-1 had low read counts in RNAseq analysis and consequently, showed no significant values for TPM after normalization. It is conceivable that certain proteins could be used in the formation of complete enveloped virions and some only in the formation of viral capsid and tegument that no envelop is present (e.g., spleen). Additionally, the MDV genes do not necessarily function as their counterparts in HSV-1 genome. The transcripts for some of the expected glycoproteins like gC, gM, gN, and gH were either not detected or expressed at very low level. It needs to be reminded that the data obtained RNA-seq is broad and relative and the sensitivity is based on the depth of reads and/or the length of targeted genes. The depth of read in our study was between 19 M and 67 M. This could explain the reason for not detecting genes with low transcript numbers.

To provide further insights into the expression pattern of viral genes in the skin of infected birds, we employed Real-Time PCR for comparative analysis of gene expression between the skin and spleen tissues of infected birds of both MD-susceptive (7_2) and resistant birds (6_3). The spleen is a secondary lymphoid organ that maintains lymphocytes and initiates an adaptive immune response. Unlike skin and FFE, no enveloped virus particles are generated within the spleen or other internal organs. The overall pattern of Real-Time PCR-based gene expression analysis in the skin of the susceptible line (7_2) showed minimal transcriptional activities for almost all the tested genes at 10dpi (latent infection) followed by a significant up-regulation of the genes at 20dpi (Reactivation phase of MDV), and a drop in the expression levels at 30dpi (Table 3 and Supplementary data 1). This pattern of gene expression closely followed the pattern of viral replication in the skin of the infected chickens. The same pattern of expression was observed in the skin of the birds of resistant line but with much reduced level of

Table 3
Real-Time PCR analysis of MDV gene expression in the skin of infected birds.

Gene ID	Fold changes with P values							
	Fold change 20 vs 10 dpi Line 7-2	P value 20 vs 10 dpi Line 7-2	Fold change 30 vs 10 dpi Line 7-2	P value 30 vs 10 dpi Line 7-2	Fold change 20 vs 10 dpi Line 6-3	P value 20 vs 10 dpi Line 6-3	Fold change 30 vs 10 dpi Line 6-3	P value 30 vs 10 dpi Line 6-3
MDV005	10.29	< 0.0001	8.91	p < 0.0001	3.27	p < 0.0001	1.39	p = ns*
MDV010	3.45	p = 0.0002	1.74	p = 0.0236	1.90	p = 0.0004	1.32	p = ns
MDV013	5.88	p < 0.0001	2.59	p = 0.0142	1.96	p = 0.0015	1.29	p = ns
MDV014	6.27	p < 0.0001	2.96	p = 0.0071	1.94	p = 0.0013	1.27	p = ns
MDV015	5.19	p < 0.0001	1.95	p = 0.0459	2.90	p < 0.0001	1.37	p = ns
MDV015.5	4.82	p < 0.0001	2.48	p = 0.0163	1.87	p = 0.0020	1.09	p = ns
MDV016	4.09	p < 0.0001	1.53	p = ns	2.41	p < 0.0001	1.13	p = ns
MDV019	5.19	p < 0.0001	2.23	p = 0404	1.55	p = 0.0360	0.92	p = ns
MDV020	5.97	p < 0.0001	2.12	p = 0.0382	2.93	p = 0.0002	1.64	p = 0.0093
MDV023	1.18	p = ns*	0.11	p = ns	2.24	p = 0.0002	1.20	p = ns
MDV024	4.66	p < 0.0001	2.71	p = 0.0069	1.60	p = 0.0031	1.10	p = ns
MDV025	4.04	p < 0.0001	2.35	p = 0.0059	1.62	p = 0.0019	1.08	p = ns
MDV028	9.86	p < 0.0001	5.74	p = 0.0036	1.32	p < 0.0001	1.36	p < 0.0001
MDV029	9.56	p < 0.0001	5.13	p = 0.0056	2.14	p = 0.0033	1.37	p = ns
MDV030	3.52	p < 0.0001	1.87	p = 0.0217	1.67	p = 0.0042	1.02	p = ns
MDV031	4.13	p = 0.0002	2.22	p = 0.0214	1.96	p = 0.01	1.28	p = ns
MDV034	4.42	p = 0.0002	5.25	p = 0.0127	1.71	p = 0.0037	1.12	p = ns
MDV037	4.08	p = 0.0002	2.08	p = 0.0294	1.45	p = ns	0.95	p = ns
MDV038	4.90	p = 0.0005	1.37	p = ns	2.85	p = 0.0001	1.36	p = ns
MDV039	4.64	p < 0.0001	2.15	p = 0.0298	1.66	p = 0.0085	1.01	p = ns
MDV040	4.82	p < 0.0001	2.55	p = 0.0115	1.61	p = 0.0122	1.12	p = ns
MDV044	3.89	p < 0.0001	2.22	p = 0.0124	1.65	p = 0.0018	1.13	p = ns
MDV047	5.16	p < 0.0001	3.18	p = 0.0004	1.68	p = 0.0013	1.18	p = ns
MDV048	3.91	p < 0.0001	2.22	p = 0.0159	1.57	p = 0.0085	1.01	p = ns
MDV049	4.37	p < 0.0001	2.45	p = 0.0136	2.15	p = 0.0002	1.54	p = ns
MDV053	3.90	p < 0.0001	1.74	p = 0.0406	2.93	p < 0.0001	1.54	p = 0.007
MDV056	3.83	p < 0.0001	2.07	p = 0.0117	1.69	p = 0.0028	1.06	p = ns
MDV059	5.80	p < 0.0001	2.42	p = 0.0081	2.11	p = 0.0004	1.29	p = ns
MDV062	4.37	p < 0.0001	1.99	p = 0.0260	2.95	p < 0.0001	1.68	p = 0.0057
MDV067	4.71	p < 0.0001	2.11	p = 0.0195	1.74	p = 0.002	0.97	p = ns
MDV068	5.17	p < 0.0001	2.30	p = 0.0098	1.80	p = 0.0016	1.11	p = ns
MDV071	3.21	p < 0.0001	1.94	p = 0.0125	1.65	p = 0.0004	1.16	p = ns
MDV072.5	2.55	p < 0.0001	1.51	p = ns	1.83	p = 0.0002	1.41	p = ns
MDV076	1.50	p = ns	0.83	p = ns	5.36	p = 0.0004	1.68	p = 0.0015
MDV088	4.34	p < 0.0001	2.64	p = 0.0008	1.69	p = 0.0011	1.17	p = ns
MDV089	3.86	p < 0.0001	1.96	p = 0.0423	2.46	p < 0.0001	1.27	p = ns
MDV090	5.31	p < 0.0001	2.02	p = ns	1.86	p = 0.0014	1.16	p = ns
MDV093	6.71	p < 0.0001	3.70	p < 0.0001	1.83	p = 0.0046	1.19	p = ns
MDV095	7.78	p < 0.0001	4.11	p = 0.0008	1.93	p = 0.0007	1.27	p = ns
MDV096	4.48	p < 0.0001	1.72	p = ns	2.84	p < 0.0001	1.49	p = ns
RLORF4.2	5.77	p = 0.0011	1.91	p = 0.0341	2.68	p = 0.0004	1.22	p = ns

* P = ns: P value not significant.

transcriptional activities (Table 3 and Supplementary data 2). With some minor exception, the expression levels of most of genes in the spleen of line 7_2 were comparable to those of line 6_3 (Table 4 and Supplementary data 3 and 4). The viral telomerase gene (*vTR)*, that was detected in the RNA-seq analysis, was the only gene that could not be amplified in the skin of the susceptible line using Real-Time PCR.

In the spleen tissues of both lines, most genes exhibited higher level of expression at 10dpi, followed by reduced transcriptional activities at 20 and 30dpi. The differential expression observed between skin and spleen is due to the fact that in visceral organs, including spleen, viruses enter a latency phase of infection around 10dpi followed with minimal viral gene activity for up to two weeks. Higher expression levels of genes observed at 10dpi in the Real-Time PCR analysis, could be the residual transcriptional activities of the viral genes during the lytic cycle.

It is not clear, however, that the three infection phases of MDV life cycle (lytic, latency, and reactivation) exist in the skin and FFE. The comparative analysis of the expression of a few genes between the skin tissues of the susceptible and resistant lines at all three time-points are depicted in Fig. 2. Similar comparison of two randomly selected genes between the skin and the spleen tissues of both lines are shown in Fig. 3.

It should be mentioned that although the general pattern of gene expression between the RNA-seq and Real-Time PCR analyses were similar, the comparison was not without disagreement. As mentioned, the RNA-seq provides a broad scope of gene expression profiling and detection of a specific transcript depends on depth of reads and the length of targeted genes. Real-Time PCR analysis, on the other hand, is an extremely sensitive technique that will amplify the smallest number of transcripts within a sample and provide a more accurate analysis of gene expression pattern.

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Ethical statement

All animal experiments were approved and carried out in accordance to the guidelines set forth by the ADOL Institutional Animal Care and Use Committee and the Guidelines for Care and Use of Laboratory Animals published by Institute for Laboratory Animal Research (ILAR Guide) in 1996 (http://www.nap.edu/openbook.php?

Table 4
Real-Time PCR analysis of MDV gene expression in the spleen of infected birds.

Gene ID	Fold changes with P values							
	Fold change 20 vs 10 dpi Line 7-2	P value 20 vs 10 dpi Line 7-2	Fold change 30 vs 10 dpi Line 7-2	P value 30 vs 10 dpi Line 7-2	Fold change 20 vs 10 dpi Line 6-3	P value 20 vs 10 dpi Line 6-3	Fold change 30 vs 10 dpi Line 6-3	P value 30 vs 10 dpi Line 6-3
MDV005	0.93	p = ns*	1.13	p = ns	1.47	p = ns	1.59	p = ns*
MDV010	0.29	p < 0.0001	0.30	p < 0.0001	0.55	p < 0.0001	0.46	p < 0.0001
MDV013	0.25	p < 0.0001	0.35	p < 0.0001	0.67	p = 0.0292	0.59	p < 0.0001
MDV014	0.26	p < 0.0001	0.64	p < 0.0001	0.58	p < 0.0001	0.65	p = 0.0013
MDV015	0.23	p = 0.0005	0.29	p = 0.0009	0.58	p < 0.0001	0.41	p < 0.0001
MDV015.5	0.28	p < 0.0001	0.33	p < 0.0001	0.62	p < 0.0001	0.40	p < 0.0001
MDV016	0.23	p = 0.0022	0.21	p = 0.0020	0.59	p < 0.0001	0.57	p < 0.0001
MDV019	0.48	p < 0.0001	0.66	p = ns	0.63	p = ns	0.64	p = ns
MDV020	0.27	p < 0.0001	0.24	p < 0.0001	0.48	p < 0.0001	0.47	p < 0.0001
MDV023	0.34	p = 0.0061	0.47	p = 0.0172	0.80	p = 0.0029	0.58	p = 0.0053
MDV024	0.40	p < 0.0001	0.56	p < 0.0001	0.60	p = 0.0029	0.89	p = ns
MDV025	0.44	p < 0.0001	0.58	p = 0.0005	0.72	p = 0.0002	0.68	p = 0.0169
MDV028	0.44	p < 0.0001	0.58	p = 0.0004	0.77	p = 0.0055	1.92	p = 0.0065
MDV029	0.39	p < 0.0001	0.55	p < 0.0001	0.57	p < 0.0001	0.43	p < 0.0001
MDV030	0.22	p < 0.0001	0.26	p < 0.0001	0.49	p < 0.0001	0.40	p < 0.0001
MDV031	0.22	p < 0.0001	0.23	p < 0.0001	0.47	p < 0.0001	0.42	p < 0.0001
MDV034	0.55	p = 0.0004	0.71	p = 0.0110	0.84	p = 0.0418	0.81	p = ns
MDV037	0.62	p < 0.0001	1.16	p = ns	1.10	p = ns	0.66	p = ns
MDV038	0.41	p < 0.0001	0.49	p < 0.0001	0.72	p < 0.0001	0.71	p = 0.0008
MDV039	0.34	p < 0.0001	0.43	p < 0.0001	0.55	p < 0.0001	0.63	p = 0.0016
MDV040	0.30	p = 0.0013	1.77	p = ns	0.93	p = ns	0.90	p = ns
MDV044	0.44	p < 0.0001	0.70	p = 0.0017	0.74	p < 0.0001	0.66	p = 0.0109
MDV047	0.42	p < 0.0001	0.79	p = 0.0234	0.66	p < 0.0001	0.68	p = ns
MDV048	0.58	p < 0.0001	1.12	p = ns	1.01	p = ns	0.75	p = ns
MDV049	0.41	p < 0.0001	0.75	p = 0.0132	0.61	p < 0.0001	0.50	p = 0.0004
MDV053	0.34	p = 0.0152	0.44	p = 0.0299	0.70	p < 0.0001	0.65	p = 0.0053
MDV056	0.31	p < 0.0001	0.39	p < 0.0001	0.56	p < 0.0001	0.58	p = 0.0001
MDV059	0.34	p < 0.0001	0.35	p = ns	0.71	p = ns	0.70	p = ns
MDV062	0.34	p = 0.0101	0.47	p = 0.0255	0.70	p < 0.0001	0.70	p = 0.0119
MDV067	0.48	p = 0.0005	0.59	p = 0.0005	0.77	p < 0.0001	0.64	p = 0.0034
MDV068	0.41	p < 0.0001	0.54	p = 0.0002	0.73	p = 0.0367	0.77	p = 0.0149
MDV071	1.08	p = ns	1.12	p = ns	0.48	p < 0.0001	0.34	p < 0.0001
MDV072.5	0.28	p < 0.0001	0.26	p < 0.0001	0.56	p < 0.0001	0.42	p < 0.0001
MDV076	1.28	p = 0.0334	1.40	p = 0.0067	1.91	p = 0.0297	1.71	p = ns
MDV088	0.23	p < 0.0001	0.25	p < 0.0001	0.56	p < 0.0001	0.52	p < 0.0001
MDV089	0.26	p < 0.0001	0.25	p < 0.0001	0.58	p < 0.0001	0.40	p < 0.0001
MDV090	0.22	p < 0.0001	0.30	p < 0.0001	0.54	p < 0.0001	0.43	p < 0.0001
MDV093	0.25	p < 0.0001	0.33	p < 0.0001	0.72	p = 0.0003	0.82	p = 0.0459
MDV095	0.26	p < 0.0001	0.34	p < 0.0001	0.61	p < 0.0001	0.77	p = 0.0448
MDV096	0.30	p < 0.0001	0.39	p < 0.0001	0.68	p < 0.0001	0.67	p = 0.0128
RLORF4.2	1.37	p = ns	1.01	p = ns	0.98	p = ns	1.35	p = ns
VTR 1	0.08	p = ns	0.22	p = ns	0.73	p = ns	1.11	p = ns

* P = ns: P value not significant.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2019.109882.

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