

CYTOKINE DETECTION IN EIAV-INFECTED EQUINE MONOCYTE-DERIVED
MACROPHAGES USING QUANTITATIVE REAL-TIME POLYMERASE CHAIN
REACTION

A Thesis

by

CHARLOTTE ANNETTE ALLEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

December 2007

Major Subject: Veterinary Microbiology

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ABSTRACT

Cytokine Detection in EIAV-Infected Equine Monocyte-Derived Macrophages
Using Quantitative Real-Time Polymerase Chain Reaction. (December 2007)

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The replication of equine infectious anemia virus (EIAV) in macrophages not only leads to cell death, but also to the induction of a variety of cytokines that may affect immune function. Cytokine production may be responsible for the fever, anorexia, hemorrhages, lethargy or thrombocytopenia seen in the acute and chronic phases of equine infectious anemia (EIA). The study of the equine immune system and inflammatory responses, by measuring cytokine expression, can provide important insight into disease pathogenesis in the horse. We have extended studies of virulent and avirulent EIAV clones by examining the effects of Env proteins on cytokine expression in equine monocyte-derived macrophages (EMDM) using EIAV₁₇, EIAV₁₉, EIAV_{17SU}, and EIAV_{17TM} viruses. In the current studies a set of quantitative real-time polymerase chain reaction (QPCR) assays for the equine cytokines IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α were validated using QPCR primers and probes which were generated for the aforementioned equine genes.

DEDICATION

To my family

ACKNOWLEDGEMENTS

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NOMENCLATURE

ACD	anticoagulant citrate dextrose
AIDS	acquired immunodeficiency syndrome
BIV	bovine immunodeficiency virus
CA	capsid
CAEV	caprine arthritis encephalitis virus
Cf2Th	canine thymus
CM	control media
CPE	cytopathic effect
Cpm	counts per minute
Ct	threshold cycle
CV	coefficient of variation
ED	equine dermis
EIA	equine infectious anemia
EIAV	equine infectious anemia virus
ELR-1	equine lentivirus receptor-1
EMDM	equine monocyte-derived macrophages
Env	envelope
FEK	fetal kidney cells
FIV	feline immunodeficiency virus
Gag	group-associated antigen
Hpi	hours post-infection
HIV	human immunodeficiency virus
IP3	inositol 1,4,5 triphosphate
L	late
LPS	lipopolysaccharide
LTR	long terminal repeat

MA	matrix
MCP-1	monocyte chemoattractant protein
MDBP	methylated DNA-binding protein site
MEM α	minimum essential medium alpha
mAb	monoclonal antibodies
MVV	maedi-visna virus
NC	nucleocapsid
NX[S/T]	N-linked glycosylation sites
PBMC	peripheral blood mononuclear cells
PND	principal neutralizing domain
Pol	polymerase
PR	protease
PU.1	purine-rich element
QPCR	quantitative real-time polymerase chain reaction
R	repeat
RAO	recurrent airway obstruction
RRE	rev-response element
RT	reverse transcriptase
RT-cPCR	reverse transcription competitive chain reaction
RT-PCR	reverse transcription polymerase chain reaction
RPA	ribonuclease protection assay
SCID	severe combined immunodeficiency
SIV	simian immunodeficiency virus
SU	surface
TAR	trans-activation responsive
TM	transmembrane
U3	unique 3 end
U5	unique 5 end

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CHAPTER I

INTRODUCTION

1.1. EIAV classification

Equine infectious anemia virus (EIAV), a lentivirus belonging to the family *Retroviridae*, affects horses, ponies, donkeys and mules. The family gets its name from the fact that these viruses contain the reverse transcriptase (RT) enzyme which enables them to reverse the flow of genetic information, from RNA to DNA whereas the conventional flow of genomic information in most other living organisms is from DNA to RNA (Dahlberg, 1988). Based on reverse transcriptase activity, genetic organization, serologic cross-reactivity and ultrastructure, EIAV is most closely related to maedi-visna virus (MVV) of sheep and caprine arthritis encephalitis virus (CAEV) of goats (Gonda, et al., 1978; Stephens et al., 1986; Kawajami et al., 1987; Montelaro et al., 1993). EIAV is also related other nonhuman lentiviruses such as bovine immunodeficiency virus (BIV) and feline immunodeficiency virus (FIV). One major difference between EIAV and other lentiviruses is that EIAV does not encode a Vif protein (Oberste and Gonda, 1992; Olsen, 2001; Navarro and Landau, 2004). EIAV is also distantly related to the two human immunodeficiency viruses (HIV-1 and HIV-2), which cause acquired immunodeficiency syndrome (AIDS) and the simian immunodeficiency virus (SIV) (Gonda, et al., 1978; Stephens et al., 1986; Kawajami et al., 1987; Montelaro et al., 1993). HIV and SIV are closely related to each other and differ from the other lenti-

This thesis follows the style of Journal of Immunological Methods.

viruses by the use of CD4 protein as receptor on lymphocytes and the absence of dUTPase (Olsen, 2001).

1.2. EIAV morphology and genome organization

EIAV is an enveloped RNA virus approximately 100 nm in diameter covered with surface “knobs” (Gonda, et al., 1978). The structure of a mature EIAV virion consists of a conically shaped core containing viral RNA surrounded by an exterior lipid envelope that is derived from the host-cell membrane during budding (Gonda, et al., 1978) (Fig.1.1, Fig.1.2).

All retroviruses including EIAV contain three major genes: *gag* (group-associated antigen), *pol* (polymerase), and *env* (envelope) (Fig. 1.3) (Olsen, 2001; Balvey et al., 2007). EIAV *gag* encodes the capsid proteins, which include the membrane-interacting matrix (MA) p15, the capsid (CA) p26, and the RNA-binding nucleocapsid (NC) proteins p11 and p9 (Stephens et al., 1986; Hussain et al., 1988). *Pol* encodes protease (PR), reverse transcriptase (RT), dUTPase and integrase (Leroux et al., 2004). *Env* encodes two glycoproteins, the surface glycoprotein (SU) and the transmembrane glycoprotein (TM) (Leroux et al., 2004). The EIAV genome also contains additional open reading frames encoding the Tat, Rev, and S2 proteins (Fig. 1.3) (Leroux et al., 2004).



Fig. 1.1. Electron micrograph of thin sections of equine macrophages infected with EIAV from the pER lineage. Arrow indicates budding and mature extracellular particles at 31,000-fold magnification. Micrograph courtesy of Dr. Fred Fuller.

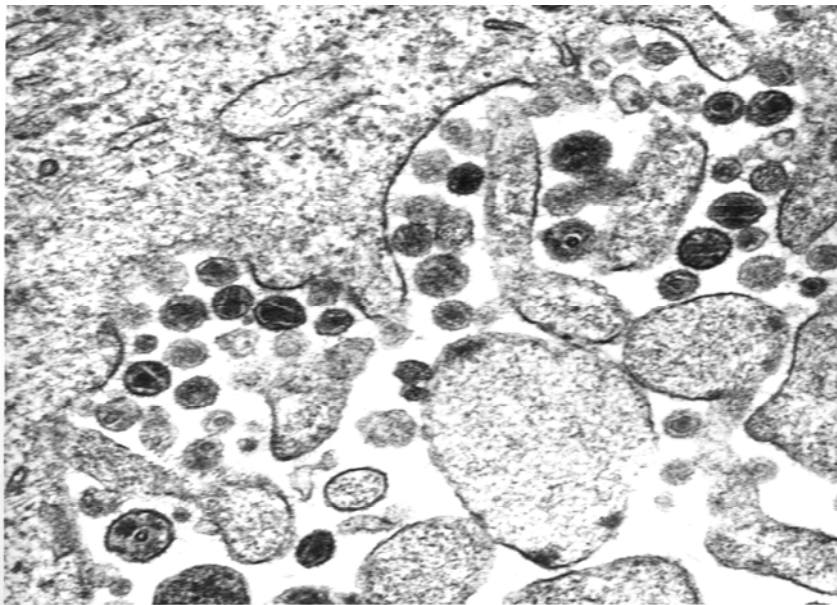


Fig.1.2. Electron micrograph of mature EIAV virus particles at a 38,000-fold magnification. The virus was derived from the pER lineage, and was adapted to replicate in non-macrophage cell types as well as macrophages. Micrograph courtesy of Dr. Fred Fuller.



Fig.1.3. Diagram showing the location of the three major genes *gag*, *pol*, and *env* in addition to the accessory genes *tat*, *rev*, and *S2* on the EIAV genome. Courtesy of Dr. Susan Payne.

1.2.1. Structural proteins

1.2.1.1. Gag

Full length viral mRNA is used to synthesize Gag polyproteins in the cytoplasm (Vogt et al., 1975; Puffer et al., 1998). The Gag polyproteins are then transported to the plasma membrane, where they assemble to form immature budding particles (Vogt et al., 1975; Puffer et al., 1998). The assembly of the Gag precursor proteins at the plasma membrane plays a crucial role in virus budding and release from the host cells (Leroux et al., 2004). The binding of the Gag polyprotein to the host cell membrane induces the assembly and budding of retroviruses (Swanstrom and Willis, 1997). Small regions of Gag, namely L (Late) domains also drive the final step of separation of the emerging viral particle (Swanstrom and Willis, 1997). During or shortly after budding, cleavage of the EIAV Gag-precursor (Pr55^{gag}) polyprotein by the viral protease generates the four major internal structural proteins of the mature virion: p15 matrix (MA), p26 capsid (CA), p11 and p9 nucleocapsid (NC) proteins (Hussain et al., 1988; Stephens et al., 1986). After the Gag polyprotein is cleaved into four different proteins, MA remains

associated with the inner face of the viral membrane, and CA forms a shell by condensing around the NC/RNA complex forming a mature infectious virion (Freed, 1998).

1.2.1.2. Pol

Various enzymatic activities are performed by *pol* gene products produced by the cleavage of the EIAV Gag-Pol precursor (Pr180^{gag/pol}) (Leroux et al., 2004). These activities include reverse transcriptase-RNaseH (p66), which is needed for the synthesis of DNA from viral RNA; a protease (p12) essential for the processing of the polyproteins Gag and Gag-pol during budding; a dUTPase (p15) necessary for EIAV replication in non-dividing monocytes-derived macrophages; and integrase needed for integration of the provirus (Threadgill et al., 1993; Lichtenstein et al., 1995; Steagall et al., 1995).

1.2.1.3. Env

The EIAV *env* gene is similar in organization to that of other retroviruses (Leroux et al., 2004). The *env* gene encodes the SU (gp90) and TM (gp45) glycoproteins that become incorporated in the virus envelope (Leroux et al., 2004). The glycoproteins SU and TM are virus specific surface “knobs” that are required for virus penetration of host cells and act as potent immuno-stimulants (Parekh et al., 1980). SU contains over three times as many N-linked glycosylation sites (NX[S/T]) as TM, based on the deduced amino-acid consensus sequence of EIAV_{PV} (Leroux et al., 1997). This may explain the enhanced ability of SU to stimulate the production of antibodies (Leroux et al., 1997). Based on binding assays from other lentiviruses, SU may interact

with equine lentivirus receptor-1 (ELR1), the newly identified cellular receptor for EIAV (Leroux et al., 2004; Ball et al., 2005; Zhang et al., 2005).

During the course of infection and recurring disease, SU evolves rapidly (Hussain et al., 1987; Payne et al., 1989; Leroux et al., 1997). Based on a study of antigenic variation within the *env* gene, SU can be divided into variable and conserved regions (Payne et al., 1987). Based on amino acid sequence alignments of SU, variation is not random but is restricted to eight well-defined variable regions (V1 to V8) (Ball et al., 1992; Zheng et al., 1997a,b; Leroux et al., 1997).

The role of mutations in the variable regions of SU was studied using monoclonal antibodies (Hussain et al., 1987). Monoclonal antibodies that reacted with variable epitopes on SU effectively neutralized the virus, whereas the conserved epitopes of SU and TM reacted primarily with non-neutralizing antibodies (Hussain et al., 1987; Payne et al., 1989). Studies using monoclonal antibodies identified three linear neutralizing epitopes on SU (Hussain et al., 1987; Ball et al., 1992). Of the three neutralizing epitopes found, two consisted of amino acid residues within the V1 region (181-210). V1 of EIAV is thought to correspond to the principal neutralizing domain (PND) or V3 in the surface unit of glycoprotein gp120 of HIV-1, as this region of EIAV SU has the potential to form a loop configuration stabilized by disulfide bonds (Ball et al., 1992; Cook et al., 1995). Thus, three separate EIAV SU domains (PND, hypervariable region and the region between) may be capable of inducing neutralizing antibodies (Zheng et al., 1997a).

Previous studies have suggested that the extracellular domain of TM is not likely to play a role in neutralization sensitivity (Cook et al., 1995). The carboxy terminus of TM from SIV and HIV are shown to influence cytopathology, growth kinetics and viral infectivity (Chakrabarti et al., 1989; Lee et al., 1989; Chirmule et al., 1990; Rice et al., 1990; Miller et al., 1991; Beisel et al., 1993). The discovery of the Ttm, a protein encoded by the first exon of *tat* and a portion of the carboxy terminus of the TM protein-coding region, could lead the discovery of a role in viral infectivity, growth kinetics, and cytopathology for the carboxy terminus of TM (Beisel et al., 1993).

1.2.2. Non-structural proteins

Unlike the genomes for the primate lentiviruses HIV and SIV, which encode for six additional genes, the EIAV genome only has the capacity to encode three additional small accessory proteins originally designated S1, S2, and S3 (Leroux et al., 2004; Das and Jameel, 2005; Levy, 2006). The EIAV S1 accessory protein is now commonly referred to as the EIAV transactivator protein (Tat) because it has a similar function to HIV Tat (Leroux et al., 2004). The EIAV S2 accessory protein does not correspond in homology to other known lentiviral proteins (Leroux et al., 2004). The EIAV S3 accessory protein corresponds to HIV-1 Rev (Leroux et al., 2004).

1.2.2.1. Tat

The transactivator of transcription (*tat*) gene is located between the *pol* and *env* genes of EIAV (Dorn and Derse, 1988; Sherman et al., 1988). Tat functions to transactivate the EIAV long terminal repeats (LTR) in order to activate viral gene expression (Dorn et al., 1990; Noiman et al., 1990). EIAV gene expression is controlled

by the interaction between the EIAV Tat protein and the viral trans-activation responsive (TAR) element associated with the LTR (Leroux et al., 2004). These functions make it likely that Tat is essential for the replication of EIAV (Fagerness et al., 2006; Desrosiers, 2007).

1.2.2.2. S2

EIAV S2 gene has a similar location to HIV *vif* between *pol* and *env*, but has no amino acid sequence homology to *vif* (Li et al., 1998), a lentiviral gene important for the replication of most lentiviruses in cultured cells (Desrosiers, 2007). The S2 protein is comprised of only 65 amino-acids, but contains 3 highly conserved amino-acid motifs: a nucleoporin (GLFG) motif, a SH3 domain binding (PXXP) motif and a nuclear localization domain (RRKQETKK) motif (Li et al., 2000). The detection of antibodies directed against S2 in naturally and experimentally infected animals confirms that S2 is produced in virus infected cells (Schiltz et al., 1992; Desrosiers, 2007). One study suggests that S2 is a cytoplasmic protein that is not incorporated into viral particles (Yoon et al., 2000). The role of S2 has been the focus of several studies, but its role in viral pathogenesis still remains unclear (Li et al., 2000; Fagerness et al., 2006). S2 is not necessary for viral replication *in vitro* in a number of equine cell lines or in monocyte-derived macrophages as described in a few mutational studies involving the pathogenic molecular clone EIAV_{UK} (Cook et al., 1998; Li et al., 1998). Some of the same studies also found that S2 is not needed in the transition of latently EIAV-infected monocytes to differentiated productively infected macrophages (Li et al., 1998). But more recent studies using a molecular clone containing a mutated S2 coding region have found S2 is

an important determinant of viral replication and pathogenesis during EIAV infection of horses and ponies (Fagerness et al., 2006; Li et al., 2000). S2 deletion mutants replicated to lower levels (reduced viral RNA levels), did not induce clinical thrombocytopenia, and did not induce clinical signs when compared with the parental cloned EIAV (Li et al., 2000; Fagerness et al., 2006).

1.2.2.3. Rev

All lentiviral genomes are relatively small and therefore must utilize complex molecular mechanisms to allow differential expression of viral proteins (Leroux et al., 2004). The use of suboptimal splice sites allows lentiviruses to utilize alternative RNA splicing that leads to the production of various mRNA from a primary transcript (Leroux et al., 2004). During transcription the EIAV LTR regulates the production of full-length genomic and singly spliced mRNAs encoding the structural proteins Gag, Pol, and Env and small multi-spliced transcripts encoding the accessory proteins Tat, S2, and Rev (Rasty et al., 1990; Stephens et al., 1990). The need for unspliced or partially spliced RNA has to be met by EIAV-infected cells, since most cellular mRNA is fully spliced before export into the cytoplasm (Leroux et al., 2004). Rev (regulator of expression of viral proteins) is required for the expression of structural proteins and for virus production by allowing the translocation of unspliced and partially spliced mRNA from the nucleus to the cytoplasm (Martarano et al., 1994; Leroux et al., 2004; Fagerness et al., 2006). EIAV Rev mediates the transport of spliced and unspliced viral transcripts from the nucleus to the cytoplasm by direct interaction with a 55-nucleotide region

located near the end of the *env* gene referred to as the Rev-responsive element (RRE) (Martanaro et al., 1994; Belshan et al., 1998; Chung and Derse, 2001).

1.2.3. Long terminal repeats

EIAV transcriptional activity is regulated by the lentiviral LTR consisting of three segments U3 (unique, 3' end), R (Repeated) and U5 (unique, 5' end) (Leroux et al., 2004). The U3 region of the LTR contains elements that function as transcriptional enhancers (Payne et al., 1999). The U3 region is one of the most variable regions in the entire EIAV genome (Payne et al., 1999). This variation can be attributed to duplications in addition to deletions and nucleotide substitutions (Carpenter et al., 1991; Payne et al., 1994; Maury et al., 1997). LTR sequence variation can affect cell tropism, virulence, and replication (Payne et al., 1999; Payne et al., 2004; Hines et al., 2004; Maury et al., 2005). A variety of cellular DNA-binding factors interact with specific motifs on the LTR sequence to control the EIAV promoter (Leroux et al., 2004). The compact EIAV promoter contains an enhancer made up of four basic DNA elements: a methylated DNA-binding protein site (MDBP), which can act either as a positive or a negative transcription factor; two PEA2 elements; a PEA3/*ets* motif; and, an AP-1 site (Carvalho and Derse, 1993a,b). The *ets* binding motifs that interact with the macrophage- and B-cell-specific transcription factor PU.1 (purine-rich element 1), required for viral transcription in primary macrophages, are also contained in the enhancer portion of the LTR (Carvalho and Derse, 1993(b); Lloberas et al., 1999; Hines et al., 2004).

1.3. EIAV replication

Many nonprimate lentiviruses replicate primarily in macrophages because of the need for cellular transcription factors present in these cells (Gabuzda et al., 1989; Desrosiers, 2007). EIAV is similar to maedi-visna virus (MVV) and caprine arthritis encephalitis virus (CAEV) in that viral replication occurs in macrophages (Narayan and Clements, 1989; Sellon et al., 1992; Montelaro et al., 1993; Narayan et al., 1993). A number of studies have shown that mature tissue macrophages rather than peripheral blood monocytes are responsible for a majority of viral replication during EIAV infections (McGuire et al., 1971; Sellon et al., 1992; Oaks et al., 1998; Harrold et al., 2000). EIAV appears to target mature tissue macrophages of the spleen, liver, lymph nodes, kidney, lung, heart, brain, stomach, bone marrow, thymus, adrenals, and intestine (McGuire et al., 1971; Sellon et al., 1992; Desrosiers, 2007).

The development of macrophages begins in the bone marrow as granulocyte-macrophage progenitor cells which develop into monocytes (Ma et al., 2003; Narayan and Zink, 1988). After leaving the bone marrow, monocytes circulate in the bloodstream for several hours before settling into their target tissue where they mature into tissue-specific macrophages such as the macrophage-like type A cells that line the synovium, Kupffer cells of the liver, alveolar macrophages of the lung, or tissue macrophages in connective tissue (Ma et al., 2003; Narayan and Zink, 1988). Macrophages and monocytes are an important part of the innate immune system because they play a dual role in host defense (Ma et al., 2003). Not only do macrophages play a

key role in innate immune response, but they also act as important accessory cells in the adaptive immune response (Ma et al., 2003).

Although infection of monocytes with EIAV results in a nonproductive infection, they may serve as a reservoir and participate in the dissemination of the virus (Harrold et al., 2000). Undifferentiated monocytes latently infected with EIAV are seen as “Trojan horses” because of their potential to disseminate the virus throughout the body without detection by the host immune system (Harrold et al., 2000).

Renal macrovascular endothelial cells can also be infected with EIAV (Maury et al., 1998). Macrovascular endothelial cells may also serve as a reservoir for virus infection, at least in long-term inapparent infections (Maury et al., 1998). Another study also showed that endothelial cells are infected during acute, virulent infection (Oaks et al., 1999). Vascular endothelial cells have been identified as a new *in vivo* host cell for EIAV, however, the role of endothelial cell infection in the pathogenesis or persistence of EIAV remains to be determined (Oaks et al., 1999; Harrold et al., 2000). *In vivo* endothelial cell infection does not appear to be a determinant of virulence for EIAV, but endothelial cell infection could contribute to EIAV-associated thrombocytopenia (Oaks et al., 1999).

Some laboratory strains of EIAV, such as EIAV_{WSU5} and EIAV_{MA-1}, have been adapted to replicate to high titer *in vitro* in equine macrophages (Kono et al., 1967), endothelial cells (Maury et al., 1998), equine dermis fibroblasts (ED) (Malmquist et al., 1973; Klevjer-Anderson et al., 1979), fetal equine kidney (FEK) cells (Benton et al., 1981), and canine and feline fibroblast cell lines (Klevjer-Anderson et al., 1979; Benton

et al., 1981). Strains of EIAV previously grown in fibroblasts usually have a preference for replication in fibroblasts and are weakly virulent or avirulent *in vivo*, whereas strains obtained *in vivo* during a viremic episode or strains passaged in macrophages replicate to higher titers in primary equine macrophage cultures and are usually virulent when placed back into the horse (Carpenter and Chesebro, 1989; Perry et al., 1992).

1.4. EIAV molecular clones

In the beginning of EIAV research, cell-adapted laboratory strains derived from the Wyoming strain such as EIAV_{WSU5}, EIAV_{PR} (avirulent prototype), and EIAV_{PV} (pony virulent) were used exclusively to study EIAV. But these laboratory strains, like the primary isolate (EIAV_{Wyo}), contained a variety of genomic species. In order to study the different aspects of EIAV replication, virulence and viral pathogenesis, molecular cloning was used to obtain a more homogenous genomic population. The first full-length EIAV molecular clone (CL22-V) was developed from the Malmquist strain of EIAV (Whetter et al., 1990). This clone was a success in that it was able to replicate in both canine thymus (Cf2Th) cells and equine dermal cells, and horses infected with this clone developed an antibody response; however this clone did not cause clinical disease in horses and, therefore, was of little use in the study of virulence and viral pathogenesis (Whetter et al., 1990). Two infectious molecular EIAV clones, pSPEiav19 and pSPEiav44, were developed from avirulent cell-adapted prototype (EIAV_{PR}) Wyoming strain (Payne et al., 1994). EIAV₁₉, the virus stock derived from pSPEiav19, failed to cause disease in a Shetland pony infected model (Payne et al., 1994). Two more chimeric clones, p19/wenv16 and p19/wenv17, were generated when the 5' and 3' LTRs

and the *env* sequences of pSPeIav19 were replaced with those derived from the highly virulent Wyoming field strain EIAV (EIAV_{Wyo}) (Payne et al., 1998). p19/wenv17 plasmid was used to produce the virus stock EIAV₁₇ that reproducibly causes acute disease in a Shetland pony model (Payne et al., 1998).

Previous studies of *in vivo* virulence have focused on the LTR and *env* regions of EIAV (Payne et al., 2004). Specifically the SU and TM regions of *env* are targeted in these studies (Payne et al., 2004). In order to study the determining factors of EIAV virulence and pathogenesis two additional EIAV clones, p17Nhe/19/wyoLTR and p19Nhe/17, were constructed to investigate the relative contribution of SU versus that of the TM/*Rev* region to the virulence phenotype of EIAV₁₇ (Payne, et al., 2004). The major difference in these clones is that p17Nhe/19/wyoLTR contains only the SU region from the virulent parent EIAV₁₇, while p19Nhe/17 contains only the TM/*Rev* region from EIAV₁₇ (Payne, et al., 2004). The virus stock generated from p17Nhe/19/wyoLTR is designated EIAV_{17SU}; the virus stock generated from p19Nhe/17 is designated EIAV_{17TM}. A commonality of these two clones is that they share common *gag*, *pol*, *tat*, and *S2* sequences with both EIAV₁₇ and EIAV₁₉, but derive the LTRs from EIAV₁₇ (Payne, et al., 2004).

1.5. EIAV transmission and pathogenesis

EIAV is the only lentivirus for which there is reasonable proof of vector-borne transmission (Desrosiers, 2007). Infection by EIAV occurs mainly by the transfer of blood between infected horses and uninfected horses by way of horseflies and deerflies that are interrupted during feeding (Hawkins et al., 1973; Hawkins et al., 1976; Kemen

et al., 1978; Issel and Foil, 1984). EIAV can also be spread through contaminated needles and instruments, blood transfusions and from mare to foal (Issel et al., 1985). The transmission of EIAV through breeding is rare (Kemen and Coggins, 1972; Tashjian, 1984). Horses can become seropositive during any stage of disease, but may remain seronegative for as long as 45 days post-infection (Coggins et al., 1972; Issel and Coggins, 1979). Like all lentiviruses, there is no effective vaccine or cure for EIAV, which results in a life-long infection (Leroux et al., 2004).

Horses infected with EIAV, whether naturally or experimentally, may manifest one or more clinical stages of the disease (Russell et al., 1998). At first exposure to EIAV, horses enter the acute stage of EIAV defined by a short period of disease that appears one week to one month post-infection and may be followed by chronic equine infectious anemia (EIA) (Issel and Coggins, 1979). Both the acute and chronic stages of EIA may be characterized by clinical signs such as fever, anorexia, hemorrhages, lethargy, or thrombocytopenia, depending on the virulence of the EIAV strain (Montelaro et al., 1993). Usually the acute stage is characterized by high fever, thrombocytopenia and occasionally anemia, while recrudescing fever, weight-loss, ventral edema, and severe anemia are seen in the chronic stage of EIA (Sellon, 1993). Hemolytic anemia may result as EIAV enters the bloodstream and antigen-antibody complexes form that associate with the surface of erythrocytes (Desrosiers, 2007). The kidneys may also be affected by the formation of antigen-antibody complexes (Desrosiers, 2007). The chronic phase of the disease, which can last up to a year or longer, is marked by recurring disease cycles (Leroux et al., 2004). The frequency of

irregular cycles of viremia and the severity of the associated clinical signs usually decline over time as the horse enters the inapparent or subclinical stage of infection (Kono, 1973). Rarely does acute or chronic EIA lead to death, unlike other lentiviruses, such as HIV, which result in a progressive degenerative disease and eventual death if left untreated (Issel and Coggins, 1979; Harrold et al., 2000).

Greater than 90% of horses infected with EIAV undergo transition into the inapparent or subclinical state and appear clinically normal with no signs of disease (Olsen 2001; Montelaro et al., 1993). In the inapparent or subclinical stage, overt clinical signs and viremia are absent for the normal life span of the horse (20 to 30 years) (Harrold et al., 2000; Montelaro et al., 1993). However, inapparent carriers do show increases in plasma total solids and serum globulin concentrations and decreases in serum albumin concentration and serum albumin-to-globulin ratio, indicating a chronic, ongoing infection (Russell et al., 1998). An inapparent or subclinical infection can be due to a number of factors such as contracting a less virulent strain of the virus or complex interactions between the horse's immune system and the virus that eventually regulate virus replication (Sellon, 1993; Olsen, 2001).

1.6. Equine cytokine production

1.6.1. Equine immune activation and dysfunction during EIAV infection

Cytokines induced by EIAV replication in the macrophage play an important role in the pathogenesis of EIA by affecting immune function and leading to the cell's destruction (Narayan and Zink, 1988; Narayan and Clements, 1989; Montelaro et al., 1993). Lesions observed during necropsy of horses with acute and chronic EIAV,

include enlargement and accentuated lobular structure of the liver, enlargement of the spleen, ventral subcutaneous edema, generalized lymph-node enlargement, mucosal and visceral hemorrhages, and vessel thrombosis (Kono, 1973; Kemen and Coggins, 1972). An accumulation of lymphocytes and macrophages in periportal areas of the liver, and in lymph-nodes, adrenal gland, spleen, meninges, and lung can be observed microscopically (Henson and McGuire, 1971; Ishii and Ishitani, 1975; McGuire, 1986). It has been hypothesized that these lymphoproliferative lesions are the result of attempts by virus-reactive T-lymphocytes to control infection (Sellon et al., 1994). Dysregulation or excessive production of cytokines by infected macrophages may result in the pathological propagation of subsets of lymphoid cells (such as cytotoxic T lymphocytes) and inhibition of others (such as helper T lymphocytes) causing these lymphoproliferative lesions (Narayan and Clements, 1989).

Following an acute episode of EIAV the hepatic lymphoproliferative lesions regress and become morphologically undetectable during the inapparent stage of EIA, but they will recur with the onset of clinical disease (Konno and Yamamoto, 1970; Henson and McGuire, 1971). Although the pathological and histological findings at necropsy are often unremarkable, inapparent carriers of EIAV may show signs of pathological changes in the kidneys and eyes (Konno and Yamamoto, 1970; Sellon et al., 1994). Some pathological changes observed in inapparent carrier horses include an increase in cellularity and thickening of glomerular tufts of the kidney, retinal depigmentations and prominent choroidal vessels in the eyes, and chronic non-

granulomatous choroiditis with foci of lymphocytic infiltrates in the eyes (Tashjian, 1984; Sellon et al., 1994).

Important insight into EIA pathogenesis in the horse can be provided by studying the equine immune system's inflammatory response, such as measuring cytokine expression in EIAV-infected macrophages. Investigating the dysregulation of cytokines secreted from EIAV-infected macrophages is important to the study of EIAV-induced pathogenesis for several reasons. EIAV replication *in vivo* may be enhanced in several ways by the secretion of pro-inflammatory cytokines such as interleukins (IL-1 α , IL-1 β , IL-6, IL-8) and tumor necrosis factor alpha (TNF- α) by macrophages (Lim et al., 2005). Like HIV-1 and SIV, EIAV may recruit uninfected monocytes to the site of viral replication through pro-inflammatory cytokine stimulation of monocyte chemotactic factors (Schmidmayerova et al., 1996; Zink et al., 2001). Pro-inflammatory cytokines may also stimulate adhesion molecules that promote monocyte migration into tissue (Sampson et al., 2002) and induce EIAV-activating molecules by nonmonocytic cells (Lim et al., 2005).

Cytokine dysregulation may coincide with clinical signs such as fever, anorexia, petechial hemorrhages, lethargy, anemia and thrombocytopenia (Montelaro et al., 1993; Lim et al., 2005). Both IL-1 and TNF- α may contribute to EIA anemia by activating macrophages and neutrophils, thus enhancing phagocytosis of complement-coated erythrocytes and possibly downregulating erythropoietin production and dysregulating iron metabolism, thereby suppressing erythropoiesis (Sentsui and Kono, 1987a,b; Swardson et al., 1992; Sellon et al., 1994; Means, 1997, 2000). Though multifaceted,

TNF- α effect on the bone marrow is largely suppressive (Tornquist et al., 1997). Megakaryocyte growth *in vitro* is also inhibited by TNF- α (Geissler et al., 1991). The injection of purified TNF- α in mice and humans suppresses the production of platelets and induces severe thrombocytopenia possibly by stimulating the secretion of platelet agonists (Michelmann et al., 1997; Tacchini-Cottier et al., 1998).

TNF- α and IL-1 also have similar inflammatory effects which include the migration of leukocytes, activation of endothelial cells, and stimulation of the hypothalamic-pituitary axis (Tracey and Cerami, 1994). IL-1, which induces the expression of prostaglandins, can have many local and systemic biological effects including fever, anorexia, and lethargy, all common signs of EIA (Dinarello, 1996). IL-1 can also induce the expression of other cytokines such as TNF- α and IL-6 (Gadient and Patterson, 1999).

There is also a positive association between TNF- α activity in serum and plasma, as measured using the WEHI 164 clone 13 cytotoxicity assay, and viremia as indicated by fever, anemia and thrombocytopenia in EIAV-infected ponies (Costa et al., 1997). The same study also showed a positive correlation between TNF- α levels and both EIAV virulence and disease enhancement in ponies immunized with virus enriched major core protein-p26; these data suggest that TNF- α may also have a pathogenic role in acute disease (Costa et al., 1997). Increases in serum TNF- α levels are seen during acute EIA infection in both immunocompetent and severe combined immunodeficiency (SCID) foals just prior to and at the onset of thrombocytopenia (Tornquist et al., 1997). These findings are not surprising in light of what is known about TNF- α effect on

immune response, which include signs of a host inflammatory response to microbial infection such as fever, anorexia and depression, all of which are clinical signs of EIA (Tornquist et al., 1997). The biological effects of TNF- α are dose-dependent: prolonged exposure to low doses of TNF- α result in severe weight loss, anorexia, and muscle wasting, whereas high doses can result in shock, tissue injury, vascular leakage, hypoxia, and other pathological features indistinguishable from septic shock (Tracey and Cerami, 1994).

Earlier studies have also shown an increase in cytokine production during EIAV *in vivo* infection, specifically IL-6, TNF- α , transforming growth factor beta (TGF- β) and interferon alpha (INF- α) (Sellon et al., 1998; Tornquist et al., 1997). IL-1 α , IL-1 β , TNF- α , and IL-6 induced by EIAV-infected macrophages are known for their pyrogenic activities (Dinarello, 1999). In addition to fever, these cytokines lead to wasting diseases commonly seen in HIV/AIDS due to anorexia and hypermetabolism (Chang et al., 1998).

A correlation between viremia and increased serum IL-6 levels in ponies infected with the virulent Wyoming strain of EIA also supports the link between viral replication, EIAV pathogenesis, and pro-inflammatory cytokines (Sellon et al., 1998). Viral stimulation of IL-6 production may contribute to the clinical disease seen in these ponies such as the development of fever (Helfgott et al., 1989), hypergammaglobulinemia and polyclonal B cell activation (McGuire et al., 1971; Russell et al., 1998). IL-6 also inhibits the production of IL-1 and TNF- α *in vivo* (Gadient and Patterson, 1999).

Chronic immune activation and dysfunctional cytokine production has been known to occur in other lentivirus-infected macrophages (Lechner et al., 1997; Legastelois et al., 1998; Fantuzzi et al., 2003). The secretion of proinflammatory cytokines IL-1, IL-6, IL-8, and TNF- α , as well as, IL-10 and INF- β have been linked to HIV infection of macrophages at all stages of HIV infection (Gessani et al., 1994; Fantuzzi, et al., 2000; Alfano and Poli, 2002). Although an increase in IL-8 expression has not been associated with EIAV infection, macrophages infected *in vitro* with CAEV have shown an increase in both IL-8 and monocyte chemoattractant protein 1 (MCP-1) expression while those infected with MVV have shown an increase in IL-8, IL-1 β , and TNF α (Lechner et al., 1997; Legastelois et al., 1998). Enhanced IL-8 expression during CAEV infection in goats may also be responsible for the inflammation of the radiocarpal joints leading to arthritis, and the inflammation of other tissues such as the mammary gland, lungs, and brain (Lechner et al., 1997). Further evidence of a correlation between cytokine dysregulation, lentivirus infection and pathogenesis can be seen in MVV infection in sheep where an increase of IL-8, a CXC chemokine, may contribute to lung disease (Legastelois et al., 1998). The lung disease seen with experimental or natural infection MVV is characterized by interstitial pneumonia with mural and luminal alveolitis consisting mainly of macrophages, neutrophils, and lymphocytes (Cordier et al., 1990; Cordier et al., 1992), together with lymphocytic nodules and zones of smooth muscle hyperplasia (Mornex et al., 1994).

In more recent EIAV studies, equine monocyte-derived macrophages (EMDM) infected with the virulent molecular clone EIAV₁₇ showed increases in IL-1 α , IL-1 β , IL-

6, IL-10 and TNF- α mRNA levels as early as 0.5 to 1 hour post-infection (hpi) (Lim et al., 2005). These findings suggest that both *in vitro* and *in vivo* viral replication and thus EIAV pathogenesis may be modulated by pro-inflammatory cytokines (Lim et al., 2005). Lim et al. (2005) also reported different cytokine responses by virulent and avirulent EIAV clones, in particular the up regulation of mRNA expression of IL-1 α , IL-1 β , TNF- α , IL-6, and IL-10 *in vitro* infection of EMDM with the virulent EIAV₁₇ clone. It is still unknown what factors influence cytokine induction and EIAV virulence. Follow-up studies need to be done to determine whether, SU or TM, found in the *env* region, is the determining factor in cytokine induction and virulence. The four chimeric molecular clones EIAV₁₇, EIAV₁₉, EIAV_{17SU}, and EIAV_{17TM} mentioned earlier should be useful for these follow-up studies.

1.7. Quantitative real-time polymerase chain reaction assay validation

1.7.1. Introduction to quantitative real-time polymerase chain reaction

The study of the equine immune system inflammatory response to EIAV has been restricted by the lack of reagents for measuring cytokine expression. Comprehensive immunoassay studies have been hindered by the fact, with the exception of TNF- α (Vick et al., 2007), that the expression of equine IL-1 β , IL-6, and TGF- β can only be detected using antibodies specific to human or other animal cytokines (Billinghurst et al., 1995; Charan et al., 1997; Rodriguez et al., 1996). The measurement of equine cytokine mRNAs is an alternative to immunoassays for the detection of cytokine expression (Cherwinski et al., 1987). Various methods including reverse transcription competitive polymerase chain reaction (RT-cPCR), reverse transcription

polymerase chain reaction (RT-PCR), Southern blot techniques, quantitative real-time polymerase chain reaction (QPCR), and the ribonuclease protection assay (RPA) have been used to detect equine cytokine mRNA expression (Grünig and Antczak, 1995; Rottman et al., 1996; Byrne et al., 1997; Swiderski et al., 1999; Leutenegger et al., 1999; Giguère and Prescott 1999; Garton et al., 2002; Boyd, et al., 2003; Lim, et al., 2003; Lim et al., 2005; Murphy et al., 2007; Vick et al., 2007).

QPCR is the most sensitive and accurate of the methods used to quantify the mRNA expression of cytokines, which are often expressed at very low levels (Wang and Brown, 1999; Blaschke et al., 2000). QPCR (also known as real-time PCR, real-time quantitative PCR [RTQ-PCR]) became commercially available in 1997 (Isono, 1997; Wittwer et al., 1997). QPCR, which is based on fluorescence-kinetic RT-PCR, allows the quantification of the PCR product in “real-time” by measuring PCR product accumulation during the exponential phase of the reaction (Blaschke et al., 2000; Giuliatti et al., 2001). Either a fluorescent dye (e.g., SYBR-Green or ethidium bromide, Higuchi et al., 1992) or labeled, sequence-specific probes (e.g., TaqMan®, Heid, et al., 1996) can be used to monitor the simultaneous amplification and detection of specific DNA sequences (Giuliatti et al., 2001). QPCR offers many advantages over traditional RNA blot methods/techniques which include increased sensitivity and speed (Giuliatti et al., 2001). Not only is it much faster than previous RT-PCR methods, QPCR is less labor-intensive and lacks post-PCR processing of products, thus there is a high throughput with a reduced risk of contamination (Giuliatti et al., 2001). It also provides ultra-rapid cycling, which means it only takes 30 minutes to 2 hours to run an assay

(Blaschke, 2000). QPCR also has a wide dynamic range of up to eight orders of magnitude and it requires 1000-fold less RNA than some conventional assays (Heid et al., 1996). It is also capable of detecting a 2-fold difference between samples; thus, it is specific, sensitive, reproducible, and not much more expensive than conventional PCR (<http://www.dorak.info/genetics/realtime.html>, 2007).

1.7.2. Development of quantitative real-time polymerase chain reaction validation protocol

In order to perform additional studies on induction of cytokines by EIAV, a set of QPCR assays for the equine cytokines IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α were validated using QPCR primers and probes which were generated for the equine IL-1 α , IL-1 β , IL-6, IL-8, TNF- α and 18S genes. It was important to validate the QPCR assays for equine cytokines because previous publications describing primer/probe sets for equine cytokines did not include information regarding reproducibility (intra- and inter-assay variability), efficiency, and specificity of the assays (Giguère and Prescott, 1999; Garton et al., 2002; Fumuso et al., 2003; Murphy et al., 2007; Vick et al., 2007). Assays for the equine cytokines of interest are not commercially available in the TaqMan FAM/MGB probe format, and validated primers and probes are essential in studying the pathology of EIA as well as other equine diseases.

After the primer/probe pairs were designed using Applied Biosystem's criteria, the efficiency and reproducibility of the assays were determined. The primer/probe pairs were evaluated using five criteria: 1) the efficiency of the assays; 2) the inter-assay variation; 3) the intra-assay variation; 4) the amplification specificity of the assays; and,

5) the variation in RNA extraction and cDNA synthesis. The efficiency of the primer/probe pairs should be between 90-100% (http://www.stratagene.com/techttoolbox/calc/qpcr_slope_eff.aspx, 2007). At a 100% efficiency the amplicon will double at each cycle (Gibson et al., 1996; Yuan et al., 2006).

$$E = 10^{(-1/\text{slope})} - 1 \quad (1)$$

The efficiency is derived from the slope of a standard curve in which the threshold cycle is graphed against the log of the copy number (see equation 1). The slope of the line should fall between -3.1 to -3.6 (<http://www.dorak.info/genetics/realtime.html>, 2007). The threshold cycle (Ct) corresponds to the cycle number at which the fluorescence generated within a reaction crosses the threshold (<http://www.dorak.info/genetics/realtime.html>, 2007). It is inversely correlated to the logarithm of the initial copy number (<http://www.dorak.info/genetics/realtime.html>, 2007). Thus, the Ct value assigned to a particular sample reflects the point during the reaction at which a sufficient number of amplicons have accumulated (<http://www.dorak.info/genetics/realtime.html>, 2007).

The reproducibility of the assays was measured both by the inter-assay variation (the variation between runs performed on different days) and the intra-assay variation (the variation within an assay performed on the same day). The coefficient of variation (CV), which is used to compare the variation across two sets of data, was used to measure the relative variability of the assays. The coefficient of variation is derived from the ratio of the sample standard deviation to the sample mean multiplied by 100 (see equation 2).

$$CV = \frac{\sigma}{\mu} \times 100\% \quad (2)$$

The specificity of the assays was insured by performing gel electrophoresis and DNA sequencing on the products of the cytokine assays. Bands corresponding to the target genes were seen and no non-specific amplification products were present.

1.8. Hypothesis and objectives

Fever, thrombocytopenia, hemorrhages, anorexia, and lethargy or seen in the acute and chronic phases of EIA may be due to the dysregulation or excessive production of cytokines by infected EIAV-infected macrophages. Lim et al. (2005) reported different cytokine responses by virulent and avirulent EIAV. In the current studies a set of recently described QPCR assays were used for the detection of equine cytokines IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α to examine the effects of the EIAV Env proteins on cytokine expression by using chimeric viruses with different combinations of SU and TM.

It was hypothesized that an increase in cytokine expression would be seen in equine macrophages infected with the virulent phenotype EIAV₁₇ as compared to equine macrophages infected with the avirulent phenotype EIAV₁₉. Thus, there would be a positive correlation between virulence phenotype and an increased cytokine expression in equine macrophages. It was also hypothesized that an increased cytokine expression would be seen in equine macrophages infected with EIAV_{17SU} as compared to equine macrophages infected with EIAV_{17TM}. Thus, the SU region of the *env* gene would have a greater influence on EIAV virulence and cytokine induction than the TM region.

The specific objectives of this study were:

1. To establish and validate cytokine assays using QPCR assays for the detection of equine cytokines IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α ;
 - a.) Validate primers and probes for equine IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α ;
 - b.) Validate technique for isolating RNA and amplifying cDNA;
2. To measure cytokine cDNA levels of IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α in LPS-stimulated and unstimulated EMDM using QPCR;
3. To measure cytokine cDNA levels of IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α in EMDM infected with either virulent EIAV₁₇ or avirulent EIAV₁₉ using QPCR; and,
4. To measure cytokine cDNA levels of IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α in EMDM infected with EIAV_{17SU} and EIAV_{17TM} using QPCR.

CHAPTER II

VALIDATION OF QUANTITATIVE POLYMERASE CHAIN REACTION ASSAYS
FOR MEASURING CYTOKINE EXPRESSION IN EQUINE MACROPHAGES***2.1. Introduction**

The lack of reagents for measuring cytokine expression has limited the study of the equine immune system and the understanding of inflammatory responses and disease pathogenesis in the horse. With the exception of TNF- α (Vick et al., 2007), specific monoclonal antibodies (mAb) and cytokine standards for the equine are very limited, and the expression of equine IL-1 β , IL-6, and TGF- β can only be measured using antibodies specific to human or other animal cytokines. This has impeded extensive studies using immunoassays (Billinghurst et al., 1995; Charan et al., 1997; Rodriguez et al., 1996). An alternative to immunoassays for the detection of cytokine induction is the measurement of equine cytokine mRNAs (Cherwinski et al., 1987). Methods that have been used to measure the expression of equine cytokine mRNAs include reverse transcription competitive polymerase chain reaction (RT-cPCR), reverse transcription polymerase chain reaction (RT-PCR), Southern blot techniques, quantitative real-time polymerase chain reaction (QPCR), and the ribonuclease protection assay (RPA) (Grünig and Antczak, 1995; Rottman et al., 1996; Byrne et al., 1997; Swiderski et al., 1999; Leutenegger et al., 1999; Giguère and Prescott 1999; Garton et al., 2002; Boyd et

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al., 2003; Lim, et al., 2003; Lim et al., 2005; Murphy et al., 2007; Vick et al., 2007).

Previous studies that have explored the production of equine cytokines in macrophages using RT-cPCR and QPCR techniques (Giguère and Prescott, 1999; Garton et al., 2002; Fumuso et al., 2003; Murphy et al., 2007; Vick et al., 2007) lack information regarding reproducibility (intra- and inter-assay variability), efficiency and specificity of the assays. While it is likely that the absence of validation data in some reports was due to limited manuscript lengths, it remains difficult to evaluate the utility of individual assays for diverse studies. Therefore the main objective of our study was to develop and clearly evaluate a set of QPCR assays for the equine cytokines IL-1 α , IL-1- β , IL-6, IL-8, and TNF- α . We describe primer and probe pairs for the listed cytokines that are sensitive, robust, and highly reproducible. These assays were used to measure the effects of lipopolysaccharide (LPS) stimulation on equine monocyte-derived macrophages (EMDM) as a baseline for future studies.

2.2. Methods

2.2.1. Primer design

Custom TaqMan® Gene Expression Assays of primer and probe sets for IL-1 α , IL-1 β , IL-6, IL-8, TNF- α , and 18S were designed using the Applied Biosystem's Assays-by-DesignSM Service for Gene Expression Criteria. Table 2.1 lists the sequences of the forward and reverse primers and the probe. The accession numbers for each gene are as follows: equine IL-6: U64794, AF041975, AF005227; equine IL-8: AY184956, CD53673, CD536703; equine IL-1 α : CD466534, U92480; equine IL-1 β : U92481, D42165, D42147; equine TNF- α : M64087, AB035735. Available nucleotide sequences

for each cytokine were compared, and those with target regions that were fully conserved, free of base ambiguities, and were not within alternatively spliced regions were selected and submitted to ABI for primer and probe development. All primer pairs were designed to produce amplicons smaller than 150 bp. To prevent the amplification of genomic DNA, the primers were placed in consecutive exons with the probe spanning the junction between exons. The 18S rRNA sequence M10098, AJ311673 was specifically chosen to be as universal as possible (Schmittgen and Zakrajsek, 2000; Aerts et al., 2004; Robinson et al., 2007) and to fit the parameters of the ABI 7500 Sequence Detection System (Applied Biosystems). The 18S rRNA primers and probe were designed using the Applied Biosystem's Assays-by-DesignSM Service, in order to be compatible with the TaqMan FAM/MGB probe format.

2.2.2. TaqMan primer and probe validation

Primer efficiency was determined using standard curves generated from 10-fold dilutions of plasmids encoding the transcript of each gene of interest. Plasmids containing IL-1 α , IL-1 β , and IL-8 sequences were obtained from the Pratt Laboratory, Plant Biology Department, University of Georgia (<http://www.fungen.org/Projects/Horse/Equus%20Project.htm>). IL-6 and TNF- α cDNAs were generated by standard RT-PCR and cloned using mRNA isolated from LPS-stimulated EMDM. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen Inc. Valencia, CA), and plasmid concentration was determined by spectrophotometry. Dilutions were prepared in water such that the final copy number in the QPCR assay ranged from 10 to 10⁵, and QPCR was preformed in triplicate. QPCR using plasmid standards with the addition of cDNA

derived from unstimulated equine macrophages was also performed in order to evaluate primer efficiency in the presence of background cDNA. An additional measurement of efficiency of equine cytokine assays was performed by using 2-fold serial dilutions of cDNA generated from LPS-stimulated EMDM. QPCR was performed in triplicate using the five cDNA dilutions to measure the efficiency of the five equine cytokine assays and the 18S housekeeping gene.

Table 2.1
Primers and probe combinations for quantitative real-time PCR

IL-6 f	5'-GAAAAAGACGGATGCTTCCAATCTG-3'
IL-6 r	5'-TCCGAAAGACCAGTGGTGATTTT-3'
IL-6 p	5'-CAGGTCTCCTGATTGAAC-3'
IL-8 f	5'-GCCACACTGCGAAAACCTCA-3'
IL-8 r	5'-GCACAATAATCTGCACCCACTTTG-3'
IL-8 p	5'-ACGAGCTTTACAATGATTTC-3'
IL-1α f	5'-CAATATCTTGCGACTGCTGCATTAA-3'
IL-1α r	5'-CTCTTCTGATGTATAAGCACCCATGT-3'
IL-1α p	5'-ACGCAGTGAAATTT-3'
IL-1β f	5'-TGTACCTGTCTTGTGGGATGAAAG-3'
IL-1β r	5'-GCTTTTCCATTTTCCTCTTTGGGTAA-3'
IL-1β p	5'-CCTACAGCTGGAGACAGT-3'
TNF-α f	5'-TTCTCGAACCCCAAGTGACAAG-3'
TNF-α r	5'-GCTGCCCTCGGCTT-3'
TNF-α p	5'-ATGTTGTAGCAAACCC-3'
18S f	5'- AAACGGCTACCACATCCAA-3'
18S r	5'-TCGGGAGTGGGTAATTTGC-3'
18S p	5'-AAGGCAGCAGGCGC-3'

Sense primers are designated by (f), antisense primers by an (r), and the probe is designated by a (p). Probes are labeled with 5' 6FAM, fluorescent dye 6-carboxyfluorescein, and 3' MGBNFQ, a minor groove binder/non-fluorescent quencher.

Inter-assay variability was measured using three separate QPCR assays performed on three different days. Intra-assay variability was measured by performing a single QPCR assay using 10 replicates of each of the following plasmid copy numbers 1×10^5 , 1×10^3 , 1×10^2 , and 1×10^1 . Analysis of the results included determination of the coefficient of variation (CV), the mean coefficient of variation, and the amplification efficiency.

The specificity of amplification was determined by performing gel electrophoresis and ethidium bromide staining on the QPCR products for each gene of interest to confirm product size and to detect the presence of any non-specific amplification products. The amplified DNA obtained from each primer pair was then purified using StrataPrep PCR Purification Kit (Stratagene, La Jolla, CA) and sequenced using BigDye® Terminator v1.1 (Applied Biosystems, Foster City, CA) on an ABI PRISM® 3100 Genetic Analyzer.

2.2.3. Quantitative real-time polymerase chain reaction assay

PCR reactions contained Custom TaqMan® Gene Expression Assays Mix (containing primers and probe), TaqMan® Universal PCR Master Mix (containing Taq, dNTPs and buffer), No AmpErase® UNG (final concentration 1X), and plasmid DNA in a volume of 20 μ l. The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI 7500 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50°C, 10 min at 95.0°C, followed by 40 cycles of 15s at 95.0°C and 1 min at 60.0°C.

2.2.4. Isolation of peripheral blood mononuclear cells and establishment of equine monocyte-derived macrophage cell cultures

EMDM cultures were established with methods adapted from Raabe et al. (1998). Whole blood in the volume of 500ml was collected from EIAV-negative horses into an anticoagulant citrate dextrose (ACD) solution blood collection bottle (The Metrix Company, Dubuque, Iowa 52002, USA) and centrifuged at 700 x g for 20 min to produce buffy coats. EMDM were separated by density gradient centrifugation using HybriMax histopaque ($d = 1.077\text{g/cm}^3$) cushion (Sigma, St. Louis, MO). The EMDM were washed four times with Dulbecco's Ca^{2+} - and Mg^{2+} - free PBS (Sigma, St. Louis, MO), 5% adult horse serum (endotoxin and EIAV tested, Invitrogen/Gibco, Carlsbad, CA) and 1% penicillin-streptomycin solution (Sigma, St. Louis, MO). The EMDM were diluted in complete minimum essential medium alpha (MEM α) medium supplemented with 10% adult horse serum (Invitrogen, Carlsbad, CA) to a beginning concentration of 5×10^6 cells/cm², and 5 ml aliquots were added to 25 cm² cell culture flasks and incubated at 37 °C in 5% CO₂. The next day (day 2), cells were washed to remove the non-adherent cells. Cells were incubated for an additional 7 days to allow macrophages to mature. All reagents used for EMDM cultures were tested by the manufacturer for endotoxin and certified ≤ 0.6 EU/mL endotoxin. In order to confirm the purity of the macrophage cultures, cell differentials were performed on cytospin preparations collected at days 1, 3 and 7 using 100 μ l samples. Cytospin preparations were stained with Wright's-Giemsa and 100-cell differentials were performed. Based on the

differentials the cell cultures were composed of 95% or greater macrophages at days 1, 3 and 7.

2.2.5. Stimulation of equine monocyte-derived macrophage cells with lipopolysaccharide

LPS at 10ng/ml (Chen et al., 2003) or a negative control using complete MEM α medium was added to the cell cultures on day 7 of culture and incubated for 1 h at 37 °C in 5% CO₂. Culture medium containing LPS or negative control was decanted, and cells were lysed by the addition of buffer RLT/ β -mercaptoethanol (Qiagen Inc., Valencia, CA). Lysates were used for RNA preparation and cDNA synthesis to examine differences in cytokine expression between the stimulated and unstimulated cells.

2.2.6. RNA isolation

RNA was extracted from EMDM cultures using the Qiagen RNeasy® Mini Kit (Qiagen Inc., Valencia, CA) according to manufacturer instructions for isolation of total RNA from animal cells, with the exception that 1200 μ l of buffer RLT/ β -mercaptoethanol (Qiagen Inc., Valencia, CA) was added to 25 cm² cell culture flasks for cell lysis. All RNA extractions were treated with 1 μ l of DNase I Amp Grade (Invitrogen, Carlsbad, CA) for 15 min and then heated for 10 min at 65°C. RNA isolated from the EMDM cultures was used for cDNA synthesis.

2.2.7. cDNA synthesis

First-strand cDNA was synthesized from total RNA using SuperScript™ III First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to manufacturer instructions in a 20 µl volume containing 200 U/µl SuperScript III RT, 1 µg of total RNA, 50 ng/µl random hexamers, 10 mM dNTP mix, and DEPC-treated water. The reaction was carried out for 10 min at 25°C followed by 50 min at 50°C and was terminated at 85°C for 5 min. cDNA, synthesized from the total RNA, was amplified using the ABI 7500 Real-Time PCR System in order to determine cytokine expression using quantitative real-time PCR. Relative quantification of the target was done using the signal from 18S M10098, AJ311673, a housekeeping gene, in each sample. Raw data was analyzed using the $2^{-\Delta\Delta C_t}$ method also known as the comparative threshold cycle (Ct) or $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001; Sabek et al., 2002), and the value for each sample was normalized using 18S as described in Applied Biosystems User Bulletin No. 2 (P/N 4303859) (<http://docs.appliedbiosystems.com/pebi/docs/04303859.pdf>,1997).

2.3. Results

2.3.1. Assay validation

In order to determine the efficiency, repeatability, and reproducibility of the custom gene expression assays, five sets of plasmid DNA were analyzed using IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α primers and probes. Amplification was linear over the range of 10 to 10⁵ input copies of plasmid for all five of the equine genes. A representative curve (IL-6) is shown in Fig. 2.1. The correlation coefficients of the

standard curves ranged from 0.9985-0.9993 (Table 2.2). The standard curve efficiencies of the primers and probes ranged from 99% to 101% using a plasmid DNA template (Table 2.2). The amplification efficiency of the target genes was approximately equal to that of 18S the housekeeping gene (Table 2.2).

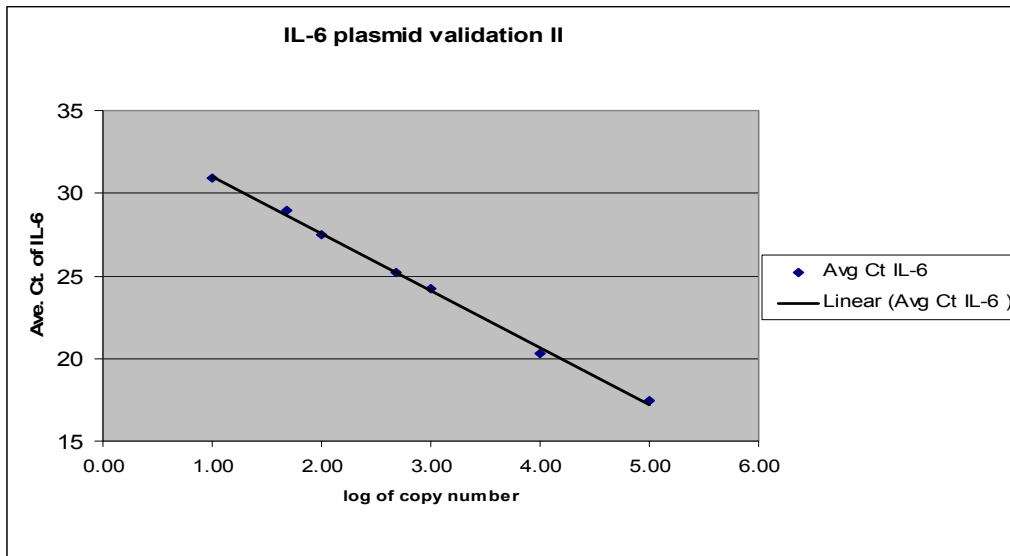


Fig. 2.1. Example of a standard curve for inter-assay validation for IL-6. On the y-axis is the average threshold cycle value for IL-6 and on the x-axis is the log of the copy number for each IL-6 plasmid dilution.

Table 2.2

Average correlation coefficients and amplification efficiencies of cytokine assays with plasmid DNA templates and cDNA dilutions

Cytokine	R²	Efficiency
IL-6	0.9985 ^a	99.0% ^b
IL-6 cDNA	0.9918 ^c	91.0% ^d
IL-8	0.9990	99.0%
IL-8 cDNA	0.9867	86.0%
IL-1 alpha	0.9985	99.7%
IL-1 alpha cDNA	0.9840	95.0 %
IL-1 beta	0.9993	101.0%
IL-1 beta cDNA	0.9819	85.0%
TNF-alpha	0.9993	99.2%
TNF-alpha cDNA	0.9959	83.0%
18S cDNA	0.9945	90.0%

Results presented as (a) and (b) are overall averages derived from data collected to determine intra-assay and inter-assay variability.

Results presented as (c) and (d) are derived from dilutions of cDNA prepared from of LPS-stimulated EMDM.

^aAverage correlation coefficients of combined assays

^bAverage efficiency of combined assays

^c Correlation coefficient from one assay run in triplicate

^d Amplification efficiencies from one assay run in triplicate

Repeatability and reproducibility of the QPCR assays were determined using intra-assay and inter-assay variation respectively. Intra-assay variation was evaluated using 10 replicates containing plasmid copy numbers of approximately 10^5 , 10^3 , 10^2 , 10^1 . The mean CVs for each cytokine are presented in Table 2.3. In no case was the intra-assay CV for any cytokine, at any quantity of input plasmid, greater than 1.8%, demonstrating the repeatability of the reactions.

The inter-assay variation was determined by performing the assays 1-5 days apart on 3 different days using dilutions containing approximately 1×10^5 , 1×10^4 , 1×10^3 , 5×10^2 , 1×10^2 , 5×10^1 , and 1×10^1 copies of plasmid. For equine IL-6 the mean CV for each dilution factor across reactions ranged from 0.2% to 0.9% with an overall mean CV of 0.5% (Table 2.3). The mean CV for equine IL-8 ranged from 0.3% to 1.1% for each dilution factor across reactions and the overall mean CV for equine IL-8 was 0.6% (Table 2.3). For equine IL-1 α and IL-1 β , the mean CV for each dilution factor across reactions ranged from 0.4% to 1.1%, and 0.2% to 1.0%, respectively. The overall mean CV for IL-1 α and IL-1 β at all copy levels was 0.7 % and 0.5%, respectively (Table 2.3). The mean CV for equine TNF- α ranged from 0.6% to 1.1%, with a mean overall CV of 0.7% at all copy levels (Table 2.3). In no case was the inter-assay CV for any cytokine, at any quantity of input plasmid greater than 1.1% demonstrating the reproducibility of the reactions. The overall mean CV across all sets of intra- and inter-assay data was 0.63% (range 0.2% to 1.8%) (Table 2.3). In Table 2.3 we also show the ranges of Ct values for lowest (10 copies) and the highest (10^5 copies) amounts of template. In no case was the Ct value for 10 copies of input plasmid greater than 35.82 (Table 2.3). The variability in Ct values did not increase at low template concentrations; the maximum range of Ct values was 2.06.

Table 2.3
Reproducibility as measured with plasmid DNA templates

Cytokine	Ct Range ^c	Ct Range ^d	Mean CV ^e
IL-6 inter-assay ^a	(17.45-17.86)	(30.51-31.07)	0.5%
IL-6 intra-assay ^b	(17.25-17.90)	(30.39-30.76)	0.6%
IL-8 inter-assay	(20.84-21.55)	(33.54-34.55)	0.6%
IL-8 intra-assay	(20.66-21.11)	(33.76-35.82)	0.8%
IL-1 α inter-assay	(18.48-18.90)	(31.73-32.93)	0.7%
IL-1 α intra-assay	(19.00-19.17)	(32.00-32.74)	0.6%
IL-1 β inter-assay	(19.87-20.29)	(32.36-33.51)	0.5%
IL-1 β intra-assay	(19.44-19.70)	(32.40-33.35)	0.6%
TNF- α inter-assay	(17.47-17.79)	(30.39-31.43)	0.7%
TNF- α intra-assay	(17.36-17.64)	(30.38-31.18)	0.7%

^aInter-assay variability was measured using three separate QPCR assays performed on three different days

^bIntra-assay variability was measured by performing a single QPCR assay

^cRange of cycle threshold for 10 copies of plasmid DNA

^dRange of cycle threshold for 10⁵ copies of plasmid DNA

^eMean of coefficient of variation

To test the efficiency of the primer and probe sets under more stringent conditions, two other assays were performed. First, cDNA from unstimulated EMDM was added to plasmid dilutions to test the efficiency of the primers and probes in the presence of a non-specific competitor. At input plasmid copy numbers of 5×10^2 to 10^5 all reactions remained linear, with amplification efficiencies similar to reactions containing only plasmid. However, at low levels of input plasmid (less than 100 copies) with the exception of IL-1 α the curves were not linear, but started to plateau, as

indicated by the arrow in the example for IL-6 (Fig. 2.2). The plateaus in the curves likely reflect the presence of cytokine mRNA in unstimulated EMDM. In the presence of equine cDNA, the linear range for IL1- β and TNF- α was from 10^2 copies to 10^5 copies and the linear range for IL-6 and IL-8 was from 5×10^2 copies to 10^5 copies. IL-1 α remained linear to 10 copies of input plasmid (essentially identical to the curves without cDNA).

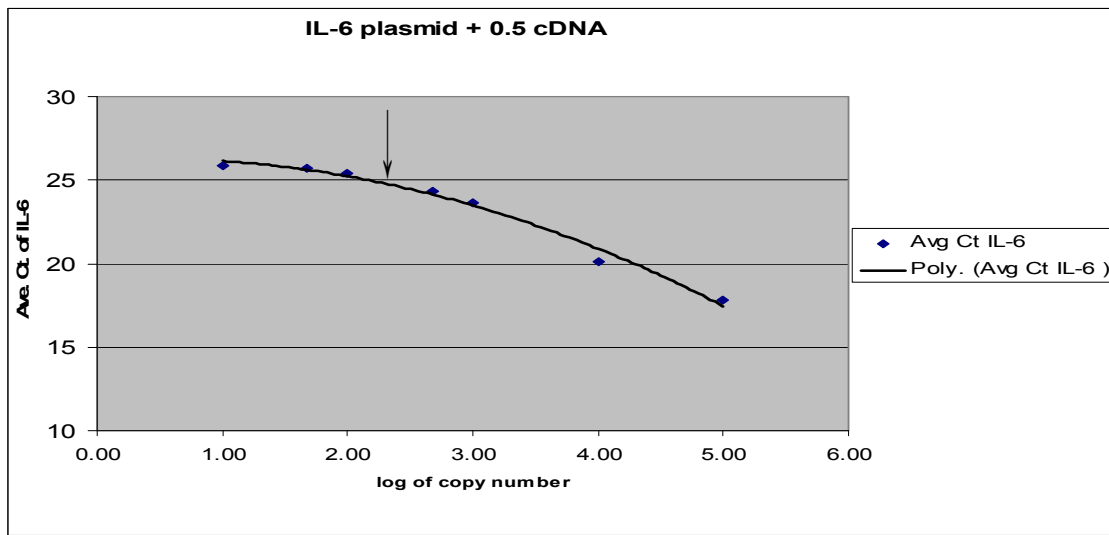


Fig.2.2. Example of a standard curve for IL-6 in the presence of background cDNA. The arrow indicates the start of a plateau.

As the addition of cDNA from equine EMDM to plasmid DNA did not allow us to examine amplification efficiencies at levels below 100 copies of input plasmid, we performed dilutions of cDNA from LPS-stimulated EMDM. In this instance we determined the efficiency of the equine cytokine gene expression assays and the 18S

housekeeping gene using a series of 2-fold dilutions (corresponding to 0.5 μ l-0.03 μ l of cDNA) from LPS-stimulated EMDM. Amplification was linear for all five cytokines and the 18S housekeeping gene and a representative curve (IL-6) is shown in Fig. 2.3. At these lower levels of input template the assays remained linear (correlation coefficients of the standard curves ranged from 0.984-0.9959) but amplification efficiencies dropped (Table 2.2). IL-1 α performed the best with an efficiency of 95%; the least efficient primer and probe was TNF- α with an efficiency of 83% (Table 2.2).

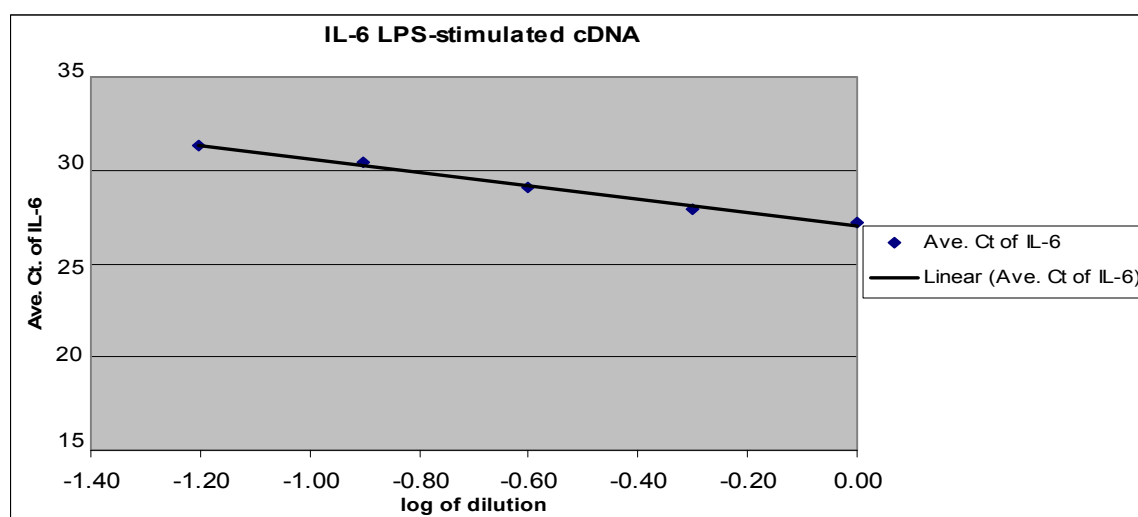


Fig.2.3. Example of a standard curve for IL-6 in the presence of LPS-stimulated cDNA.

2.3.2. Specificity of amplification of TaqMan assays

It is typically understood that TaqMan assays are specific, however to insure we had the desired product; gel electrophoresis was used to analyze the products generated from each cytokine assay. Bands corresponding to the expected sizes of the target genes

were seen, and no non-specific amplification products were present (data not shown). Each of the QPCR products was sequenced, and each product was found to be the sequence of interest (data not shown).

2.3.3. Stimulation of equine monocyte-derived macrophage cells with lipopolysaccharide

In order to provide useful comparisons for future studies three sets of cDNA from EMDM stimulated with LPS were analyzed by QPCR using IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α primers and probes. Two EMDM cultures were from the same horse, collected one month apart, and the third set was from a different horse. RNA was isolated and cDNA was prepared as described in the materials and methods section. Cytokine expression levels varied between samples, but the general trend was the same for all three samples. IL-1 α showed the greatest induction levels while IL-6 showed the lowest induction levels (Fig. 2.4). IL-1 α levels were induced approximately 300- to 30,000-fold. IL-1 β levels were induced 89-fold to 1554-fold. TNF- α levels were induced from 30-fold to 280-fold. IL-8 levels were induced from 53-fold to 133-fold. IL-6 levels were induced from 12-fold to 65-fold.

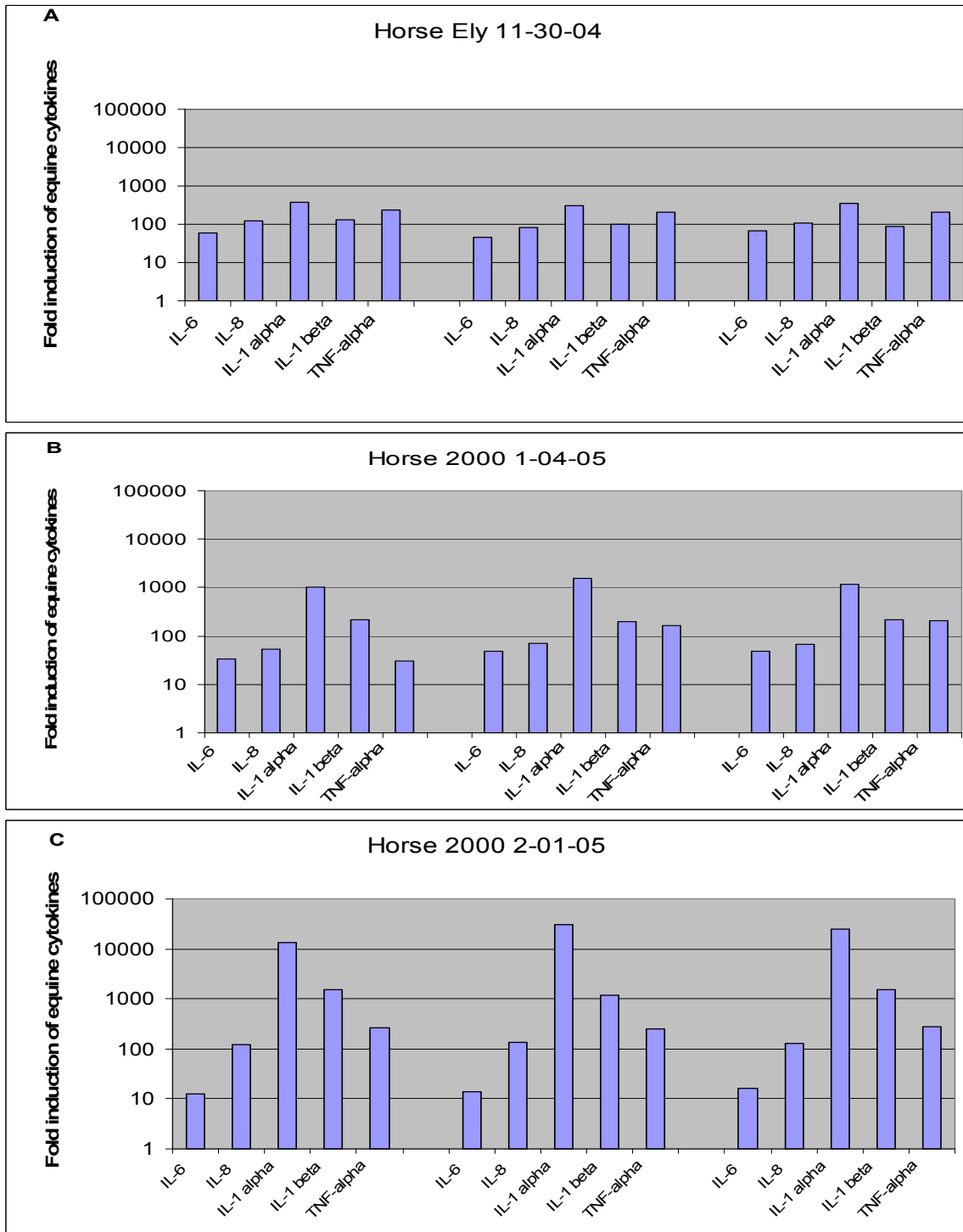


Fig.2.4. Induction of gene expression of equine cytokines after addition of 10ng/ml LPS to EMDM cells. Panel A horse Ely, Panel B & C horse 2000 1 month apart. For each bleed date one cDNA reaction was prepared. Three separate QPCR were performed (each assay performed in triplicate).

Table 2.4
Cycle threshold (Ct) values for LPS-stimulated and unstimulated EMDM

Cytokine ^a	Mean Ct ^b	Ct Range ^c	S.D. ^d	Mean CV% ^e
IL-6 unstimulated	30.36	(27.13-34.25)	2.81	9.25
IL-6 stimulated	25.89	(22.54-29.35)	2.66	10.27
IL-8 unstimulated	27.67	(27.15-28.15)	0.32	1.16
IL-8 stimulated	21.67	(19.67-23.79)	1.51	6.97
IL-1 α unstimulated	35.33	(32.58-37.65)	1.78	5.04
IL-1 α stimulated	24.85	(22.14-26.99)	2.01	8.09
IL-1 β unstimulated	31.41	(30.02-32.52)	0.98	3.12
IL-1 β stimulated	23.73	(20.39-26.77)	2.38	10.03
TNF- α unstimulated	27.85	(26.67-28.82)	0.75	2.69
TNF- α stimulated	20.81	(19.22-23.22)	1.40	6.73
18S unstimulated	7.40	(6.30-8.46)	0.58	7.84
18S stimulated	7.95	(6.93-9.66)	0.81	10.19

^a Combined statistical analysis of cycle threshold values from three different samples (two samples were from same horse taken one month apart) of unstimulated and LPS-stimulated EMDM measured using three separate QPCR assays performed on three different days.

^b Mean constant threshold values for combined data from three different samples of EMDM from two different horses.

^c Range for mean constant threshold values for combined data from three different samples of EMDM from two different horses.

^d Standard deviation of constant threshold values for combined data from three different samples of EMDM from two different horses.

^e Mean of coefficient of variation for combined data from three different samples of EMDM from two different horses.

The constant threshold (Ct) values for the three sets of cDNA from unstimulated EMDM ranged from 26.67 for TNF- α to 37.65 for IL- α (Table 2.4). The three sets of cDNA from LPS-stimulated EMDM had Ct values ranging from 19.22 for TNF- α to 29.35 for IL-6 (Table 2.4). Values for the 18S housekeeping gene ranged from 6.30 to

8.46 for cDNA from unstimulated EMDM and from 6.93 to 9.66 for cDNA from LPS-stimulated EMDM (Table 2.4). The average Ct value using cDNA from unstimulated EMDM for 18S was 7.40, the average Ct value using cDNA from LPS-stimulated EMDM for 18S was 7.95 (Table 2.4).

2.4. Discussion

QPCR is a rapid and reliable method for mRNA quantitation (Heid et al., 1996; Wang and Brown, 1999; Ficko and Černelč, 2005) however many QPCR primer and probe sets are poorly described and validation of many primer and probe sets is lacking. Although QPCR is a very specific and reproducible technique, in order for the results to be meaningful, validated primer and probe combinations must be available.

In this study we have described a set of QPCR primers and probes for quantifying equine IL-6, IL-8, IL-1 α , IL-1 β , and TNF- α , and have demonstrated that these primer and probe sets were sensitive, specific, and reproducible. Normalization to a relevant “housekeeping gene” is also important in the accuracy of QPCR. The 18S ribosomal subunit was chosen because its cellular concentration is constant within a range of 1.0- to 4.2-fold (Schmittgen and Zakrajsek, 2000; Aerts et al., 2004; Robinson et al., 2007) which correlates to a difference of 0.5-2.0 Ct values, and its amplification efficiency of 90% (Table 2.2) is similar to that of the target genes. Other housekeeping genes such as GAPDH and β -actin were considered, but were found to be inappropriate for our uses. GAPDH exhibited changes of up to 10-fold in expression in EMDM under varying conditions (J.Harrington, personal communication). Both GAPDH and β -actin are regulated within a 10-fold range and the transcription level can vary during cell

proliferation, differentiation, or activation (Schmittgen and Zakrajsek, 2000; Goidin et al., 2001; Frost and Nilsen, 2003; Aerts et al., 2004; Dheda et al., 2004). 18S and β -2 microglobulin exhibit less variability and are therefore better internal controls (Goidin et al., 2001; Aerts et al., 2004).

Primer and probe sets were initially evaluated using standard curves generated from 10-fold dilutions of double stranded circular plasmids encoding the transcript of each gene of interest. The assays were linear over the range tested (10 to 10^5 copies of plasmid) and intra-and inter-assay variation was low. Further, there was no correlation between template copy number and assay variation, suggesting that the assays are equally robust with low template amounts. When we used more biologically complex samples (dilutions of cDNA) the primers and probes maintained acceptable, albeit lower efficiencies (Gallup and Ackermann, 2006). Valid data can be obtained from efficiencies less than 90%, if amplification is linear and the amplification efficiencies of the target and reference gene are approximately equal ([http://docs.applied biosystems.com/pebiiodocs/04303859.pdf](http://docs.appliedbiosystems.com/pebiiodocs/04303859.pdf), 1997; Livak and Schmittogen 2001; Gallup and Ackermann, 2006; <http://www.dorak.info/genetics/realtime.html>, 2007).

Sensitivity of the assays was assessed using plasmid DNA dilutions. All of the assays easily detected 10 copies of input plasmid. To determine assay sensitivity for a more relevant (biologically complex) sample we added cDNA from unstimulated EMDM to plasmid dilutions. However, the addition of cDNA from EMDM to plasmid did not serve to resolve sensitivity issues because at low levels of input plasmid the curves began to plateau. It is likely that the point at which the plateau begins indicates

that the number of copies of plasmid and the number of copies of cDNA are approximately the same (between 100 and 500 copies for most of the cytokines tested). In the case of IL-6, when cDNA was added to the plasmid, the curve began to plateau at Ct values between 24 and 25 (Fig. 2.2); this corresponds reasonably well to the observed Ct values for IL-6 in unstimulated EMDM, which were as low as 27 (Table 2.4). For IL-1 α (data not shown) the curves were the same with ($R^2 = 0.9925$, amplification efficiency = 106%) and without ($R^2 = 0.9985$, amplification efficiency = 99.7%) cDNA. Ct values for IL-1 α for unstimulated EMDM alone were between 32 and 37 (Table 2.4), similar to the Ct values obtained for 10 copies of plasmid (between 31 and 32) (Table 2.3).

We did not follow up further on the issue of absolute sensitivity as it was evident that all of the assays were capable of detecting cytokine expression in unstimulated EMDM. In these experiments we generally recovered 5 μg of total RNA from 1.5- 2 x 10⁶ cells and 20 μl of cDNA was prepared from 1 μg of RNA. For all of the tested cytokine assays, amplification was positive using 0.5 μl of cDNA from unstimulated EMDM. Dilutions of cDNA prepared from LPS-stimulated EMDM revealed that as little as 0.03 μl of cDNA was sufficient to generate a detectable signal. Therefore, while we have not absolutely determined the sensitivity of these assays, they detect as little as 10 copies of plasmid and are sufficiently sensitive to detect basal levels of cytokine expression in relevant cell types. Finally we examined product specificity by gel electrophoresis and DNA sequence analysis, to demonstrate that the assays were truly specific for the desired gene.

After extensive testing, the QPCR assays were used to determine the levels of cytokine induction in LPS-stimulated EMDM. LPS was chosen for EMDM stimulation because it is a powerful activator of the innate immune system, and it is the best known and most thoroughly characterized inducer of inflammatory cytokines (Murtaugh et al., 1996). LPS induces the synthesis of a variety of cytokines and chemokines from mononuclear phagocytes; furthermore, LPS is potent inducer of both transcription and translation; a defined set of gene products, such as IL-1 α , IL-1 β , and IL-8 result from the stimulation of monocytes/macrophages with LPS (Chen et al., 2003). LPS also induces the expression of TNF- α through a wide variety of agents that activate the nuclear transcription factor, NF- κ B (Myers and Murtaugh, 1995; Chen et al., 2003).

Cytokines selected for this study, IL-6, IL-8, IL-1 α , IL-1 β , and TNF- α , are representative of the major inflammatory cytokines produced by macrophages (Murtaugh et al., 1996). Few studies have been published that evaluate cytokine production in equine macrophages stimulated with LPS. To the authors' knowledge, only three studies have used QPCR to measure cytokine production in EMDM after LPS exposure. Sykes et al. (2005) incubated equine peripheral blood mononuclear cells (PBMC) in the presence of LPS for 0 to 48 h and measured expression of TNF- α and IL-1 β mRNA. Both TNF- α and IL-1 β showed a mean increase in mRNA expression of approximately 6-fold at 6 h; thereafter expression decreased slightly but remained significantly elevated at all time points compared with time 0 (Sykes et al., 2005).

Laan et al. (2006) exposed recurrent airway obstruction (RAO) susceptible and nonsusceptible horses to aerosolized LPS, collected bronchoalveolar lavage fluid

(BALF), isolated equine alveolar macrophages at either 6 or 24 h post-exposure and measured levels of TNF- α , IL-1 β , IL-8, and IL-6 mRNA. A mean difference of approximately 7-fold was seen in TNF- α expression in RAO susceptible horses after 6 h, but had returned to baseline after 24 h (Laan et al., 2006). A significant increase was seen in IL-1 β and IL-8 expression; a mean difference of approximately 2-fold and 3-fold was seen for IL-1 β and IL-8 respectively at 6 h (Laan et al., 2006). A mean difference of approximately 4-fold was seen for IL-1 β in RAO susceptible horses at 24 h (Laan et al., 2006). However, LPS-stimulation did not significantly alter the expression of TNF- α , IL-1 β , IL-8, or IL-6 in RAO nonsusceptible horses at either 6 or 24 h (Laan et al., 2006).

The third study measured IL-8 using QPCR of equine alveolar macrophages exposed to LPS for 48 h. When compared to the untreated control, alveolar macrophages stimulated with LPS showed a mean increase of approximately 14-fold in IL-8 expression (Jackson et al., 2004).

It is not possible to make a direct comparison between this study of LPS-stimulated EMDM and the aforementioned studies due to the differences in time points and study populations. In the three cytokine studies mentioned above, TNF- α , IL-1 β , and IL-8 had an induction range of 2-to14-fold over a period of 6 to 48 h. However, the induction of cytokine gene expression upon LPS exposure of EMDM seen in this study was much greater than has been previously reported. After 1 h of LPS exposure, we observed increases in IL-1 α gene expression ranging from approximately 300- to 30,000-fold. IL-1 β mRNA levels were induced 100- to 1500-fold. TNF- α mRNA levels increased from 30- to 280-fold. IL-8 mRNA levels increased from approximately

50- to 100-fold. IL-6 mRNA levels increased from approximately 10- to 65-fold. The time points used in the other assays (earliest measurement at 6 h) could be an important factor in the difference between previously reported cytokine levels and our results.

Another likely explanation for the high levels of gene expression seen in this study is that the QPCR assays described herein are very sensitive and have a large linear range with baseline values easily detected in non-stimulated cells. The inability to accurately measure basal levels of gene expression can reduce the ability to determine fold changes, as can the use of assays with limited dynamic range. Another important aspect of this study was that great care was taken to use only endotoxin free media and PBS in our cell cultures, making the assay more sensitive to fold changes between the LPS-stimulated and unstimulated cells. The data from this study was obtained using blood from two horses taken at three different time points which produced three independent cell cultures, each assayed in triplicate, and the general trend held true for both horses. An increase of at least 10-fold was seen for IL-6, IL-8, IL-1 α , IL-1 β , and TNF- α in this study for both horses. Although the absolute increases in cytokine gene expression were different for the two study animals, the relative trend in cytokine gene expression was the same. IL-1 α showed the greatest induction levels while IL-6 showed the lowest induction levels for the two study animals.

In conclusion, we have validated a set of QPCR assays for the equine cytokines IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α , and we have demonstrated these assays are sensitive, robust, and highly reproducible. We propose that these assays are a valid way

to measure the effects of LPS stimulation on equine monocyte-derived macrophages and that our measurements can be used as a baseline for future studies.

CHAPTER III
CYTOKINE DETECTION IN EIAV-INFECTED EQUINE MONOCYTE-DERIVED
MACROPHAGES USING QUANTITATIVE REAL-TIME POLYMERASE CHAIN
REACTION

3.1. Introduction

Equine infectious anemia virus (EIAV) belongs to the genus *Lentivirus*, which means “slow virus”, within the family *Retroviridae* (Desrosiers, 2007). EIAV infects horses, donkeys and mules and results in a life-long infection for which there is no effective vaccine or cure (Leroux et al., 2004). Based on both natural and experimental infections, equine infectious anemia (EIA) may present in one or more of the following clinical stages: acute, chronic, and inapparent (Hammond et al., 2000; Leroux et al., 2004). EIAV varies from other lentiviruses in that it causes a period of acute disease that appears 7 to 30 days post-infection that can be followed by chronic EIA (Olsen, 2001). During the acute stage of the disease, the horse may remain seronegative for as long as 45 days post-infection (Coggins et al., 1972; Issel and Coggins, 1979).

The chronic phase of the disease, which can last for a year or longer, is characterized by irregular cycles of viremia and related clinical signs (Leroux et al., 2004). Depending on the virulence of the EIAV strain, fever, anorexia, petechial hemorrhage, lethargy, or thrombocytopenia may be seen in the acute and chronic phases of the disease (Montelaro et al., 1993). High fever, thrombocytopenia and occasionally anemia are usually seen in the acute phase, while recrudescing fever, weight-loss, ventral edema, and severe anemia are seen in the chronic phase of the disease (Sellon, 1993).

During the chronic stage of the disease the frequency and severity of the clinical episodes, which can last 3 to 6 days, usually decline over time (Kono, 1973; Olsen, 2001). Infrequently, however the chronic stage may progress, resulting in chronic ill-thrift and eventual death (Issel and Coggins, 1979; Harrold et al., 2000).

About 90% of horses undergo transition to an inapparent or subclinical infection in which clinical signs appear to be absent for the normal life-span of the horse (20 to 30 years), but the horse remains infected with EIAV (Harrold et al., 2000; Montelaro et al., 1993). These horses remain infective, possibly due to complex interactions between the horse's immune system and the virus that eventually regulate viral replication (Olsen, 2001). This is in contrast to other lentiviruses, such as human immunodeficiency virus (HIV), which result in a progressive degenerative disease and are fatal, when left untreated (Harrold et al., 2000).

Viral replication of EIAV, as with most lentiviruses, occurs primarily in macrophages (Montelaro et al., 1993; Narayan and Clements, 1989; Narayan et al., 1993; Sellon et al., 1992). In addition to their role as a reservoir of viral replication, macrophages also play an important role in the pathogenesis of EIA (Narayan and Zink, 1988). EIAV replication in the macrophage results in the cell's destruction, but also induces the production of a variety of cytokines that may affect immune function (Narayan and Clements, 1989).

Other lentiviruses are known to cause chronic immune activation and dysfunctional cytokine production in infected macrophages. All stages of HIV infection are linked to the secretion of proinflammatory cytokines IL-1, IL-6, IL-8, and TNF- α , as

well as, IL-10 and INF- β from macrophages (Gessani et al., 1994; Fantuzzi, et al., 2000; Alfano and Poli, 2002). Caprine arthritis encephalitis virus (CAEV)-infected macrophages show an increase in expression of IL-8 and monocyte chemoattractant protein 1 (MCP-1), while those infected with maedi-visna virus (MVV) show an increase in IL-8, IL-1 β , and TNF α (Lechner et al., 1997; Legastelois et al., 1998). Early studies of EIAV cytokine induction show an increase in IL-6, TNF- α , TGF- β and INF- α production in ponies and horses infected with EIAV (Sellon et al., 1998; Tornquist et al., 1997). Studies performed in equine monocyte-derived macrophages (EMDM) infected with the virulent molecular clone p19/wenv17 (EIAV₁₇) showed increases in IL-1 α , IL-1 β , IL-6, IL-10 and TNF- α mRNA levels as early as 0.5 and 1 hour post-infection (hpi) (Lim et al., 2005). The production of these cytokines by infected macrophages may contribute to the cytopathic effect seen in the cells (Montelaro et al., 1993).

Early laboratory strains of EIAV contained a variety of genomic species. To study EIAV replication, virulence and viral pathogenesis, a more homogenous genomic population of viruses was needed. The development of two infectious molecular clones, pSPEiav19 and p19/wenv17, has made it possible to study the effects of EIAV both *in vivo* and *in vitro*. EIAV₁₉ (Fig. 3.1) is an avirulent virus, derived from the infectious molecular clone pSPEiav19 (Payne et al., 1994; Payne et al., 2004). Shetland ponies infected with EIAV₁₉, the virus derived from clone pSPEiav19, show no clinical signs of disease (Payne et al., 1994). When the 5' and 3' long terminal repeats (LTR) and the *env* sequences of pSPEiav19 are replaced with those derived from the highly virulent Wyoming field strain EIAV (EIAV_{Wyo}) a chimeric clone, p19/wenv17 is generated

(Payne et al., 1998). EIAV₁₇, (Fig. 3.1) derived from molecular clone p19/wenv17, is a virulent virus that causes severe fever and thrombocytopenia at 5 to 8 days post-infection in Shetland ponies (Payne et al., 1998).

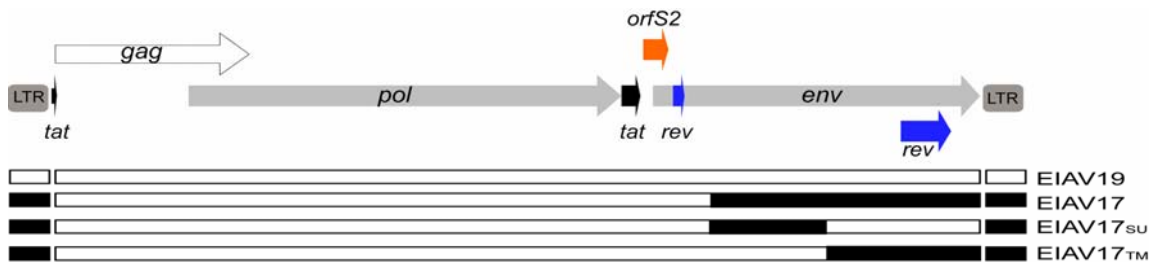


Fig. 3.1. Diagram of the four EIAV clones EIAV₁₉, EIAV₁₇, EIAV₁₇^{SU}, and EIAV₁₇TM. Courtesy of Dr. Susan Payne.

Shetland ponies experimentally infected with EIAV₁₇ develop fever and thrombocytopenia within 5 to 8 days post-infection (Payne et al., 1998), whereas ponies infected with 10-fold higher doses of EIAV₁₉ remain free of clinical signs (Payne et al., 1994). The differences in disease manifestation between EIAV₁₉ and EIAV₁₇ cannot be explained by differing levels of viral expression in cultured EMDM (Lim et al., 2005). In EMDM, both viruses replicate to high titer and induce cytopathic effects (CPE), indicating other factors are responsible for the difference in virulence (Lim et al., 2005).

A correlation between virulence phenotype and virally-induced increases of cytokine expression has recently been demonstrated in EMDM studies, using a ribonuclease protection assay (RPA) (Lim et al., 2005).

To further explore the factors that influence cytokine induction and EIAV virulence a set of additional clones, p17Nhe/19/wyoLTR and p19Nhe/17, designated EIAV_{17SU} and EIAV_{17TM}, respectively, were developed (Fig. 3.1). These clones were constructed to investigate the role of the *env* region in determining the virulence phenotype of EIAV₁₇ (Payne, et al., 2004). Because the *env* region contains both an surface (SU) and a transmembrane (TM)/*Rev* region, either one of these regions might play a greater role in virulence. Thus two new molecular clones were constructed; the first, clone p17Nhe/19/wyoLTR, contains only the SU region from the virulent parent EIAV₁₇; while the second, clone p19Nhe/17, contains only the TM/*Rev* region from EIAV₁₇ (Payne, et al., 2004). Both molecular clones have the same *gag*, *pol*, *tat*, and *S2* sequences as EIAV₁₇ and EIAV₁₉, but derive the LTRs from EIAV₁₇ (Payne, et al., 2004). The clone p17Nhe/19/wyoLTR, also referred to as EIAV_{17SU}, shares a portion of the SU region, from amino acids 147 to 444, with EIAV₁₇ (Payne, et al., 2004). Shetland ponies experience acute febrile episodes when infected with EIAV_{17SU} (Payne, et al., 2004). It should be noted that although the infecting dose of EIAV_{17SU} was up to 30-fold greater than that of the EIAV₁₇, the resulting febrile episodes were less severe than those previously observed in ponies infected EIAV₁₇ (Payne, et al., 2004).

The construct p19Nhe/17 also referred to as EIAV_{17TM} contains the SU region from EIAV₁₉ in addition to sharing some overlapping *rev* sequences and a portion of the TM region, amino acids 20 to 417 with EIAV₁₇ (Payne, et al., 2004). Although 2 ponies received 6- to 20-fold more infectious EIAV_{17TM} virus relative to the lethal dose of EIAV₁₇, the ponies infected with EIAV_{17TM} never experienced a febrile episode; their body temperatures remained normal for more than 50 days post-infection (Payne, et al., 2004). Therefore the virulence phenotype of EIAV₁₇ cannot be attributed solely to the TM/*Rev* region (Payne, et al., 2004). The ponies in this study were infected with doses of either EIAV_{17SU} or EIAV_{17TM}, equivalent to or higher than the lethal dose of EIAV₁₇, yet neither clone induced acute febrile episodes requiring euthanasia (Payne, et al., 2004).

We studied the effects of EIAV₁₇, EIAV₁₉, EIAV_{17SU}, and EIAV_{17TM} on EMDM using a set of quantitative real-time polymerase chain reaction (QPCR) assays for the equine cytokines IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α . We hypothesized that a positive correlation would be seen between the virulence phenotype of EIAV₁₇ and an increased IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α expression in EMDM. We also hypothesized that EMDM infected with EIAV₁₉ (the avirulent clone) would show little or no increase in expression of equine cytokines. We also predicted that there would be an increase in IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α expression in EMDM exposed to EIAV_{17SU} as compared to EMDM exposed to EIAV_{17TM}. It was thought that the greatest increases in expression of IL-1 α , IL-1 β , TNF- α and possibly IL-6 would be seen in EMDM exposed to EIAV₁₇ or EIAV_{17SU}.

3.2. Methods

3.2.1. Isolation and establishment of equine monocyte-derived macrophage cultures

Primary EMDM cultures were established as previously described (Allen et al., in press). Briefly, whole blood was collected from EIAV-negative donor horses into an anticoagulant citrate dextrose (ACD) solution blood collection bottle (The Metrix Company, Dubuque, Iowa 52002, USA). The blood was then centrifuged at 700 x g for 20 min to produce buffy coats, and EMDM were separated by density gradient centrifugation using HybriMax histopaque ($d = 1.077\text{g/cm}^3$) cushion (Sigma, St. Louis, MO). Dulbecco's Ca^{2+} and Mg^{2+} - free PBS (Sigma, St. Louis, MO), 5% adult horse serum (endotoxin and EIA tested, Invitrogen/Gibco, Carlsbad, CA) and 1% penicillin-streptomycin solution (Sigma, St. Louis, MO) were used to wash the EMDM four times. Complete minimum essential medium alpha ($\text{MEM}\alpha$) medium supplemented with 10% adult horse serum (Invitrogen, Carlsbad, CA) was used to dilute the EMDM to a initial concentration of 5×10^6 cells/ cm^2 . One-ml aliquots of the EMDM/ $\text{MEM}\alpha$ suspension were added to each of the 12-wells of a Poly-D-Lysine plate (Becton Dickinson Labware) and incubated at 37 °C in 5% CO_2 . Non-adherent cells were removed the following day (day 2) by washing with Dulbecco's PBS and the resulting culture of adherent cells was incubated for an additional 7 days. All reagents were manufacturer tested and certified ≤ 0.6 EU/mL endotoxin for use in EMDM cultures. It was estimated that the macrophage cultures had a 95% or greater purity based on 100-cell differentials performed on cytospin preparations stained with Wright's-Giemsa and collected at days 1, 3 and 7 using 100 μl samples.

3.2.2. Virus stocks

Molecular clones EIAV₁₇, EIAV₁₉, EIAV_{17SU}, and EIAV_{17TM} were prepared as previously described (Payne, et al., 1998; Payne et al., 2004). Viral stocks were amplified by infecting EMDM with approximately 4,000 counts per minute (cpm) of reverse transcriptase (RT) activity in 1.0 ml. Culture supernatants from infected EMDM were collected 5 to 15 days post-infection. An increase in RT activity was used to measure the virus growth. The infected culture supernatants were ultrafiltered against 500 ml fresh complete MEM α , without horse serum, using a Vivaflow 200 tangential-flow ultrafiltration unit (Sartorius Corp. Edgewood, NY) with a MWCO of 100 kDa in order to remove any cell-secreted molecules (especially cytokines molecular weights of cytokines range from 7 to 75 kDa) (Lim et al., 2005). The RT activity of the concentrated viral supernatants was remeasured after ultrafiltration. Culture supernatant of mock-infected EMDM was collected and purified under identical conditions, and was used as control medium (CM). Purified CM, EIAV₁₇, EIAV₁₉, EIAV_{17SU}, and EIAV_{17TM} contained negligible levels of endotoxin (below 0.25 EU/ml).

3.2.3. Infection of equine monocyte-derived macrophages

Cytokine profiles of infected EMDM were determined after exposure to a virulent strain of EIAV (EIAV₁₇), an avirulent strain of EIAV (EIAV₁₉), EIAV_{17SU}, and EIAV_{17TM} clones. Mock-infected EMDM was used as a negative control. At 7 days post-isolation of macrophages, EIAV₁₉, EIAV₁₇, EIAV_{17SU}, and EIAV_{17TM} (containing 30,000 cpm of RT activity in 1 ml) or 1 ml of complete MEM α medium was added to

each 12-well plate, and incubated for 1 h at 37 °C in 5% CO₂. Culture medium containing EIAV₁₉, EIAV₁₇, EIAV_{17SU}, EIAV_{17TM}, or negative control was decanted from the cells which were lysed by the addition of buffer RLT/ β-mercaptoethanol (Qiagen Inc., Valencia, CA). Lysates were used for RNA preparation and cDNA synthesis as described previously in Allen et al., (in press). At 1 hpi cells were washed with PBS and lysed by the addition of buffer RLT/ β-mercaptoethanol (Qiagen Inc., Valencia, CA). Cell lysates were stored at -80°C. The experiments were repeated in triplicate using blood collected from three different horse donors.

3.2.4. RNA isolation

As previously described in Allen et al., (in press), total RNA was isolated from EMDM cultures using the Qiagen RNeasy® Mini Kit (Qiagen Inc., Valencia, CA). Briefly, the manufacturer instructions were followed for the total isolation of RNA from animal cells. In order to eliminate any DNA contamination, the extracted RNA was treated with the addition of 1 µl of DNase I Amp Grade (Invitrogen, Carlsbad, CA) for 15 min before being heated for 10 min at 65°C. After most of the DNA was removed, the isolated RNA was used for cDNA synthesis.

3.2.5. cDNA synthesis

SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was used to synthesize first-strand cDNA from total RNA, as previously described in Allen et al., (in press). Briefly, 200 U/µl SuperScript III RT, 50 ng/µl random hexamers, 10 mM dNTP mix, and DEPC-treated water were added to 1 µg of

total RNA for a total volume of 20 μ l. The reaction conditions used were as follows: 25°C for 10 min, followed by 50°C for 50 min, and then the reaction was terminated after 5 min at 85°C. The ABI 7500 Real-time PCR System was used to amplify the resulting cDNA and determine the cytokine expression using QPCR.

3.2.6. Primer design

Primer and probe sets were designed and validated as previously described in Allen et al., (in press). Applied Biosystem's Assays-by-DesignSM Service for Gene Expression Criteria was used to design Custom TaqMan® Gene Expression Assays for IL-1 α , IL-1 β , IL-6, IL-8, TNF- α , and 18S. The following is a list of accession numbers for each gene: equine IL-1 α : CD466534, U92480; equine IL-1 β : U92481, D42165, D42147; equine IL-8: AY184956, CD53673, CD536703; equine TNF- α : M64087, AB035735; equine IL-6: U64794, AF041975, AF005227; and, the 18S housekeeping gene M10098, AJ311673. Briefly, the primers were placed in consecutive exons with the probe spanning the junction between exons to prevent the amplification of genomic DNA. In addition all primer pairs were designed to produce amplicons smaller than 150 bp. After a comparison of available nucleotide sequences, those target regions that were fully conserved, free of any base ambiguities, and not within alternatively spliced regions were submitted to ABI for primer and probe development.

3.2.7. Quantitative real-time polymerase chain reaction assay

As previously described in Allen et al., (in press), the final PCR reactions contained TaqMan® Universal PCR Master Mix (containing Taq, dNTPs and buffer), No AmpErase® UNG (final concentration 1X), Custom TaqMan® Gene Expression

Assays Mix (containing primers and probe), and cDNA sample in a total volume of 20 μ l. The PCR reactions were amplified in an automated fluorometer (ABI 7500 Sequence Detection System, Applied Biosystems), using the 96-well plate format. Amplification conditions for the reactions were 2 min at 50°C, 10 min at 95.0°C, followed by 40 cycles at 95.0°C for 15s and then a terminating step of 1 min at 60.0°C.

3.2.8. Data analysis

Relative quantification of the target in each sample was performed using the signal from 18S M10098, AJ311673, a housekeeping gene. Raw data were analyzed using the $\Delta\Delta C_T$ method, also known as the comparative threshold cycle (Ct) (Livak and Schmittgen, 2001; Sabek et al., 2002), and the value for each sample was normalized using 18S M10098, AJ311673 (Applied Biosystems User Bulletin No. 2 (P/N 4303859); <http://docs.appliedbiosystems.com/pebiiodocs/04303859.pdf>, 1997). Negative RT samples as well as samples without cDNA template were included in reactions to check for contamination. The results were presented as fold-increase in cytokine expression of cells infected with either EIAV₁₇, EIAV₁₉, EIAV_{17SU}, or EIAV_{17TM} over those of CM-treated cells.

3.3. Results

3.3.1. Expression of IL- α , IL-1 β , IL-8, IL-6 and TNF- α in equine monocyte-derived macrophages infected with EIAV clones

EMDM derived from the peripheral blood of three different horse donors was exposed for 1 h to EIAV₁₇, EIAV₁₉, EIAV_{17SU}, EIAV_{17TM}, and CM. RNA was isolated and cDNA was prepared as described in the materials and methods section. Three sets of

cDNA from EMDM exposed to the four EIAV clones and CM were analyzed by QPCR using IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α primers and probes. Fig. 3.2 shows the results, presented as a ratio of fold-increase of EIAV-treated cells over CM-treated cells, for three different horses.

Horse A showed increases in IL-1 α , IL-1 β , and TNF- α in cells exposed to EIAV₁₇, EIAV_{17SU}, and EIAV_{17TM} relative to CM, while IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α were down-regulated in cells exposed to EIAV₁₉ (Fig. 3.2). There was not a consistent pattern of induction among EIAV₁₇, EIAV_{17SU}, and EIAV_{17TM}; increases in expression varied among cytokines (Fig. 3.2).

For horse B IL-1 α , IL-1 β , and TNF- α were up regulated in cells exposed to EIAV₁₇, EIAV_{17SU}, and EIAV_{17TM} relative to CM (Fig. 3.3). Cells exposed to EIAV₁₉ demonstrated a down-regulation of IL-1 β , IL-6, and IL-8 expression (Fig. 3.3).

For unknown reasons, horse C exhibited a different pattern of induction from the other two horses. Cells exposed to either EIAV₁₇, EIAV_{17TM}, or EIAV₁₉ showed an increase in TNF- α expression, but also showed a decrease in IL-1 α , IL-1 β , and IL-6 expression (Fig. 3.4). Unlike the other two horses, there was no difference in cytokine induction between cells exposed to the virulent clone EIAV₁₇ and those exposed to EIAV₁₉ the avirulent clone (Fig. 3.4).

When the data from all three horses were averaged, a general trend emerged. Although cytokine expression levels varied between horses, the EMDM exposed to EIAV₁₇, EIAV_{17SU}, and EIAV_{17TM} showed the greatest induction levels in IL-1 α , IL-1 β , and TNF- α expression, but showed a down regulation of IL-6 expression (Table 3.1).

Looking at EMDM from individual horses, IL-1 α reached a peak induction of 7.2-fold relative to CM (Fig. 3.3). IL-1 β peaked at 7.86-fold over CM (Fig. 3.3). The highest induction of TNF- α was 4.44-fold over CM (Fig. 3.3). Insignificant induction rates were seen for IL-8 and IL-6, with 1.76-fold and 1.22-fold increases over CM respectively (Fig. 3.2 and Fig. 3.3). Unlike IL-1 α , IL-1 β , and TNF- α , it appears that IL-6 and IL-8 cDNA expression was unregulated by CM treatment.

Increases in IL-1 α (2.5-fold), IL-1 β (2.1-fold), and TNF- α (3.2-fold), relative to CM treated EMDM, were detected in EMDM treated with EIAV₁₇, (Table 3.1). EIAV₁₇-treated EMDM consistently expressed higher levels of IL-1 α and IL-1 β than EIAV₁₉-treated EMDM (Fig. 3.2, Fig. 3.3, and Fig. 3.4). Increases in expression were not seen for IL-6 or IL-8 in EIAV₁₇-treated EMDM (Fig. 3.2, Fig.3.3, and Fig. 3.4).

Increases in IL-1 α (3.6-fold), IL-1 β (3.5-fold), and TNF- α (3.1-fold) were seen in EMDM treated with EIAV_{SU}, relative to CM-treated EMDM (Table 3.1). EIAV_{17SU}-treated EMDM consistently expressed higher levels of IL-1 β and TNF- α than EIAV_{17TM}-treated EMDM (Fig. 3.2, Fig. 3.3, and Fig. 3.4). EIAV_{17SU}-treated EMDM consistently expressed higher levels of IL-1 α and IL-1 β than EIAV₁₉-treated EMDM (Fig. 3.2, Fig. 3.3, and Fig. 3.4).

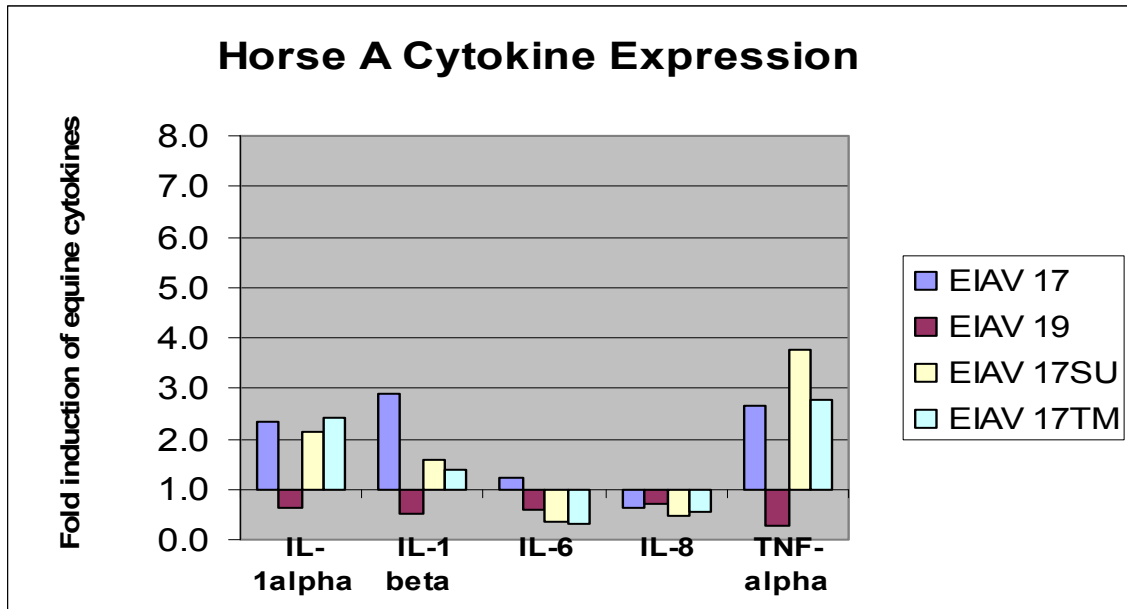


Fig. 3.2. Induction of gene expression of equine cytokines for horse A EMDM treated with either EIAV₁₇, EIAV_{17SU}, EIAV_{17TM}, EIAV₁₉ relative to CM-treated cells. Each assay was performed in triplicate.

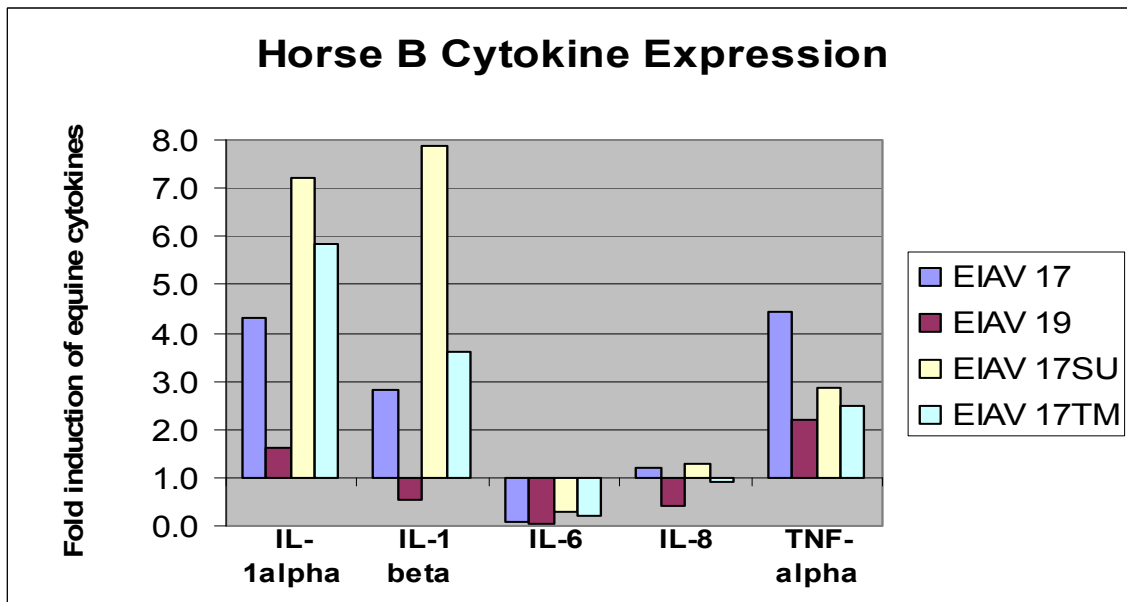


Fig.3.3. Gene expression of equine cytokines for horse B EMDM. Each assay was performed in triplicate.

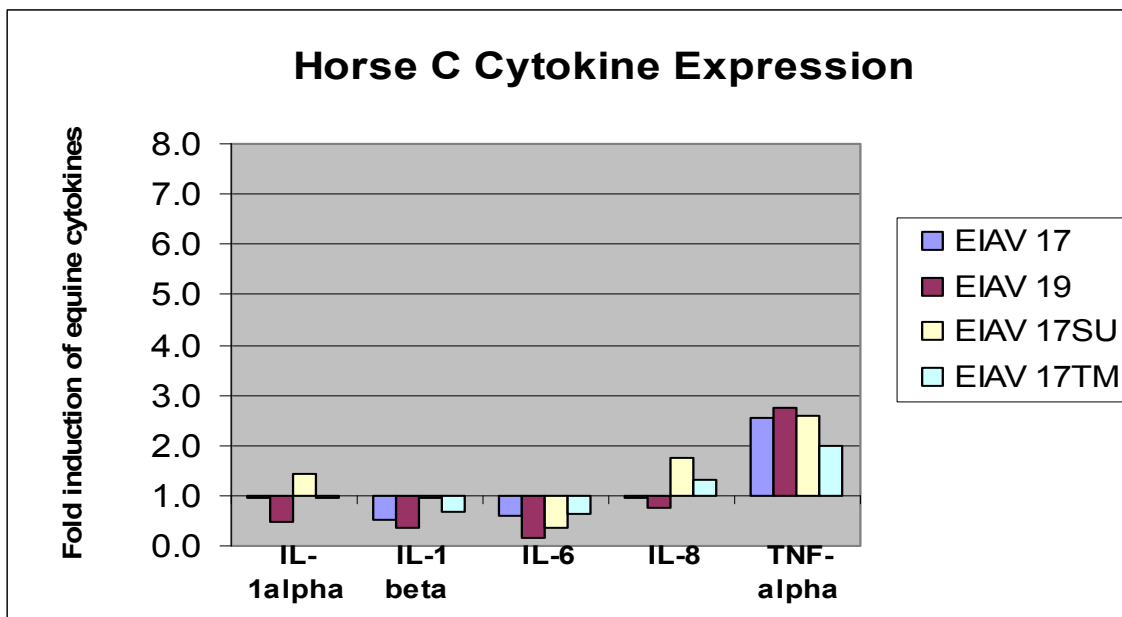


Fig. 3.4. Gene expression of equine cytokines for horse C EMDM. Each assay was performed in triplicate.

Table 3.1

Comparison of cytokine expression in EIAV₁₇, EIAV₁₉, EIAV_{SU}, and EIAV_{TM}-infected EMDM to CM-treated EMDM at 1 hpi

Cytokine	EIAV ₁₇ Ratio ^a	EIAV ₁₉ Ratio ^a	EIAV _{17SU} Ratio ^a	EIAV _{17TM} Ratio ^a
IL-1 α	2.53	0.92	3.60	3.07
IL-1 β	2.08	0.48	3.46	1.88
IL-6	0.63	0.26	0.32	0.39
IL-8	0.92	0.63	1.17	0.93
TNF- α	3.21	1.74	3.08	2.41

These results are overall averages derived from data collected from three horses.

^aAverage ratio of EIAV-infected EMDM over control media.

3.4. Discussion

In the present study we utilized a set of QPCR primers and probes for quantifying equine IL-6, IL-8, IL-1 α , IL-1 β , and TNF- α in order to investigate the dysregulation of cytokines secreted from EIAV-infected macrophages. The investigation of cytokines secreted from EIAV-infected macrophages is essential to the understanding of EIAV-induced pathogenesis for a number of reasons. Pro-inflammatory cytokines such as IL-6, IL-8, IL-1 α , IL-1 β , and TNF- α may enhance EIA pathogenesis by supporting viral replication *in vivo* (Lim et al., 2005). There is also evidence that lentiviruses such HIV-1, simian immunodeficiency virus (SIV), and possibly EIAV may use monocyte chemotactic factors stimulated by the release of pro-inflammatory cytokines from infected macrophages to recruit uninfected monocytes to the site of viral replication (Schmidmayerova et al., 1996; Zink et al., 2001). Other ways pro-inflammatory cytokines may enhance EIAV replication include the induction of EIAV-activating molecules by nonmonocytic cells (Lim et al., 2005) and the stimulation of adhesion molecules that promote monocyte migration into tissue (Sampson et al., 2002).

The cytokines IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α were chosen for this study, based on previous EIA research. Clinical signs of EIA such as fever, anorexia, petechial hemorrhages, lethargy, anemia and thrombocytopenia are thought to coincide with the secretion of these cytokines (Montelaro et al., 1993; Lim et al., 2005). Both IL-1 and TNF- α may contribute to EIA-induced anemia by suppressing erythropoiesis while enhancing phagocytosis of complement-coated erythrocytes (Sentsui and Kono, 1987a,

1987b; Swardson et al., 1992; Sellon et al., 1994; Means, 1997, 2000). EIA anemia may be further intensified by the inflammatory effects of IL-1 and TNF- α which include the stimulation of the hypothalamic-pituitary axis and the activation of endothelial cells and leukocytes (Tracey and Cerami, 1994).

Other indicators of viremia such as fever, anemia and thrombocytopenia have been linked to increased levels TNF- α activity in sera and plasma in EIAV-infected ponies (Costa et al., 1997). There is also evidence of a positive relationship between TNF- α activity in both sera and plasma and EIAV virulence and disease which suggest TNF- α may also have a pathogenic role in acute disease (Costa et al., 1997).

Another important link between viral replication, EIAV pathogenesis, and pro-inflammatory cytokines is the correlation between viremia and increased serum IL-6 levels in ponies infected with virulent EIAV (Sellon et al., 1998). The secretion of IL-6 by EIAV-infected macrophages may contribute to the clinical disease seen in these ponies, such as the development of fever (Helfgott et al., 1989), hypergammaglobulinemia, and polyclonal B cell activation (McGuire et al., 1971; Russell et al., 1998). Other studies show an increase in IL-6, TNF- α , TGF- β and INF- α production during EIAV infection *in vivo* (Sellon et al., 1998; Tornquist et al., 1997).

Although a majority of the data collected on cytokine dysregulation during EIAV infection points to the secretion of TNF- α and IL-6 by macrophages, IL-1 can have many local and systemic biological effects including fever, anorexia, and lethargy that are correlated with the clinical signs of EIA (Dinarello, 1996). While increases in IL-8 and MCP-1 expression have not been associated with EIAV infection, macrophages

infected with CAEV and MVV show increases in IL-8, IL-1 β , MCP-1 and TNF α (Lechner et al., 1997; Legastelois et al., 1998).

Increases in IL-1 α , IL-1 β , IL-6, IL-10 and TNF- α mRNA expression show up as early as 0.5 hpi in EMDM infected with the virulent molecular clone EIAV₁₇ (Lim et al., 2005). The secretion of pro-inflammatory cytokines such as IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α by infected macrophages may enhance EIAV replication *in vivo* and thus EIA pathogenesis (Lim et al., 2005).

Many questions still need to be answered about the role of EIAV virulence in producing pro-inflammatory cytokines by infected macrophages and the influence of these cytokines on EIAV replication and pathogenesis. In order to discover the factors that determine virulence, and thus EIA pathogenesis, cytokine induction was studied using four chimeric molecular clones, EIAV₁₇, EIAV₁₉, EIAV_{17SU}, and EIAV_{17TM}.

Four well-defined and biologically distinctive EIAV molecular clones were used to infect EMDM, the natural host cells for EIAV. The development of these infectious molecular clones has made it possible to study the effects of EIAV both *in vivo* and *in vitro*. The *gag*, *pol*, *tat* and *S2* sequences of EIAV₁₇ and EIAV₁₉ are identical, but these viruses differ in their long terminal repeats (LTR)s, *env* and *rev* sequences, with the regions of greatest sequence divergence occurring in SU and the U3 enhancer (Payne et al., 2004). Using chimeric clones, studies designed to define the major virulence determinants of EIAV₁₇ show that both the *env* and LTR sequences of EIAV₁₇ are required for the acute virulence phenotype (Payne et al., 2004). Replacement of either the *env* or LTRs sequences of EIAV₁₇ with those of EIAV₁₉ abolishes EIAV₁₇ virulent

effects (Payne et al., 2004). There is a difference of 30 amino acids in the SU region and 17 amino acids in the TM region of EIAV₁₇ and EIAV₁₉, giving them biological distinction (Payne et al., 2004). Further studies of EIAV₁₇ virulence determinants propose that SU plays a greater role in virulence than TM (Payne et al., 2004). EIAV₁₇ containing TM from EIAV₁₉ was shown to cause disease, whereas EIAV₁₇ containing SU from EIAV₁₉ resulted in no signs of disease (Payne et al., 2004). The role of SU as a virulence determinant is further supported by the up-regulation in mRNA expression of IL-1 α , IL-1 β , TNF- α , IL-6, and IL-10 during *in vitro* infection of EMDM by the virulent EIAV₁₇ clone, as compared to either EIAV₁₉ (an avirulent clone) or CM (Lim et al., 2005).

As further evidence of the role of SU in EIAV virulence determination, recombinant SU from virulent EIAV₁₇ (SU₁₇) induced dose-dependent diarrhea similar to that reported for SIV SU when administered intraperitoneally to mouse pups (Ball et al., 2005). Administration of SU₁₇ results in fluid accumulation in mouse intestinal loops with no histological lesions, increases in inositol 1,4,5 triphosphate (IP3) levels in HT29 cells, and induction of chloride secretory currents in Ussing chambers (Ball et al., 2005). Dose-dependent diarrhea, similar to enterotoxic peptides from SIV, was also obtained using SU₁₇₍₂₉₉₋₃₃₀₎, an SU₁₇ peptide (Ball et al., 2005). As expected, the administration of SU from the avirulent EIAV strain failed to induce a dose-dependent response in mouse pups and produced lower levels of activity than SU₁₇ in Ussing chambers and IP3 assays (Ball et al., 2005).

Because cytokines are expressed at very low levels from EIAV-infected macrophages, we chose to use QPCR. This method is one of the most sensitive and accurate methods for measuring cytokine expression in cells (Wang and Brown, 1999; Blaschke et al., 2000; Lim et al., 2005; Allen et al., in press). In order to obtain accurate results, the QPCR data were analyzed using relative quantification. Relative quantification differs from absolute quantification in that relative quantification is based on the expression levels of a target gene relative to the expression of a reference gene rather than to an external calibration curve based on known concentrations (<http://docs.appliedbiosystems.com/pebiiodocs/04303859.pdf>, 1997; Pfaffl, 2001; Livak and Schmittgen, 2001; Bubner and Baldwin, 2004). The 18S ribosomal subunit was used as the reference gene or “housekeeping gene” to normalize the QPCR results for each target gene. The 18S ribosomal subunit was chosen because it has an amplification efficiency of 90%, which is comparable to that of the primers and probes (Allen et al., in press). 18S also has a very stable cellular concentration that ranges from 1.0-fold to 4.2-fold or a difference of 0.5-2.0 Ct values (Schmittgen and Zakrajsek, 2000; Aerts et al., 2004; Robinson et al., 2007). We found that the standard deviation in LPS-stimulated and unstimulated cells ranged from 0.58 to 0.81 (Allen et al., in press). Other housekeeping genes such as GAPDH and β -actin were considered, but were found to be too variable in cellular expression (Schmittgen and Zakrajsek, 2000; Goidin et al., 2001; Frost and Nilsen, 2003; Aerts et al., 2004; Dheda et al., 2004).

The primer and probe sets for the equine cytokines IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α have been previously validated in Allen et al., (in press) using both standard

curves generated from 10-fold dilutions of plasmids encoding the transcript of each gene and dilutions of cDNA from LPS-stimulated macrophages. Non-template samples as well as negative RT samples were included in each plate as negative controls, in order to detect any evidence of DNA contamination.

We expected to see small changes in fold induction of cytokines, based on previous research by Lim et al., (2005). Although not statically significant, two of the three horses showed the expected increases IL-1 α , IL-1 β and TNF- α expression after treatment with EIAV₁₇, EIAV_{17SU}, and EIAV_{17TM} as compared to treatment with control media. Unexpectedly, we did not see increases IL-6 or IL-8 expression after treatment with EIAV₁₇, EIAV_{17SU}, and EIAV_{17TM} as compared to treatment with control media. Lim et al., (2005) also observed that IL-6 and IL-8 mRNA expression in EMDM was altered by treatment with control media. The upregulation of IL-6 and IL-8 by CM could possibly be due to a secreted factor such as prostanoids PGE₁ and PGE₂, activated protein C, or platelet-activating factor (Marshall et al., 1996).

A recent study involving murine macrophages has shown that the introduction of serum factors such as fetal bovine serum and mouse serum into macrophage cultures stimulates the production of IL-6 (Brummer et al., in press). The use of MEM α medium supplemented with 10% adult horse serum in the EMDM culture may have complicated the measurement of IL-6 production by EMDM. The production of IL-10 by virus infected EMDM could also have an effect on IL-6 production. However, the present study did not measure IL-10 expression in EIAV-infected EMDM, Lim et al., (2005) reported an increase in IL-10 expression in EMDM infected with EIAV₁₇. IL-10 is

reported to inhibit the production of IL-6 and TNF- α in mast cells stimulated with LPS, PGE₁ and PGE₂ (Marshall et al., 1996). Control media was processed the same way as the media containing the viral clones by the removal of all cell-secreted molecules under 100 kDa by filtration; but this does not completely exclude the existence of large proteins or protein complexes remaining in the control media (Lim et al., 2005). The inclusion of these large proteins or protein complexes in the control media may account for the lack of IL-6 and IL-8 expression after treatment with EIAV₁₇, EIAV_{17SU}, and EIAV_{17TM} as compared to those treated with control media.

It is also unclear why two of the horses (A and B) gave the expected trend in cytokine induction in EMDM treated with the four clones, but EMDM from the third horse (C) treated with the same clones showed little to no cytokine induction relative to control media. This unexpected result could be attributed to individual horse variation. In addition, it should be noted that the four EIAV clones were grown in EMDM obtained from horse C, and this may have down-regulated cytokine induction in the EMDM from horse C. The incorporation of host cell proteins by lentiviruses into the viral envelope can have wide ranging effects on virus life cycle, virus cell interactions, host response to virus-incorporated host-proteins, and disease pathogenesis (Cantin et al., 2005; Kolegraff, et al., 2006). We speculate that the incorporation of host cell proteins by the four EIAV clones could account for the lack of cytokine induction by EMDM obtained from horse C.

In conclusion, when all the data are taken into consideration, there were differences in cytokine expression between EMDM treated with the virulent EIAV₁₇

clone and those treated the avirulent EIAV₁₉ clone. Unfortunately, there is no clear cut evidence of a difference in cytokine induction between EMDM treated with the EIAV_{17SU} clone and those treated the EIAV_{17TM} clone. More studies need to be done using a greater number of horses in order to minimize the impact of horse-to-horse variation and perhaps to generate a clearer picture of the differences in cytokine induction between the EIAV_{17SU} clone and the EIAV_{17TM} clone.

CHAPTER IV

SUMMARY

EIAV is a lentivirus belongs to the family *Retroviridae* that infects horses, donkeys and mules and results in an enduring infection for which there is no remedy (Leroux et al., 2004). EIAV infection does not usually result in death, greater than 90% of horses transition to an inapparent or subclinical infection with the horse remaining infected for life with EIAV (Montelaro et al., 1993; Harrold et al., 2000). Because there is no vaccine or cure, horses that test positive for EIAV must be euthanized or quarantined for life in order to stop the spread of the virus (http://nahms.aphis.usda.gov/equine/equine05/equine05_infosheet_eia_trends.pdf, 2006). EIAV which has a world-wide distribution has been reduced greatly within the last 35 years, but EIAV has a high rate of mutation and the potential to once again become a problem (http://nahms.aphis.usda.gov/equine/equine05/equine05_infosheet_eia_trends.pdf, 2006).

The current study, using QPCR as a method of detection, expanded on previous reports based on an equine-specific ribonuclease protection assay (RPA) demonstrating differences in cytokine expression between EMDM treated with the virulent EIAV₁₇ clone and those treated with the avirulent EIAV₁₉ clone. Although the *gag*, *pol*, *tat* and *S2* sequences of EIAV₁₇ and EIAV₁₉ are the same, these clones differ in their LTRs, *env* and *rev* sequences, with the regions of greatest sequence divergence occurring in the region of the U3 enhancer and SU (Payne et al., 2004). Payne et al., (2004) reported that both the *env* and LTR sequences of EIAV₁₇ are required for the acute virulence phenotype, when either the *env* or LTRs of EIAV₁₇ are replaced virulence is eliminated.

The *env* gene is important to virulence because it encodes the SU (gp90) and TM (gp45) glycoproteins that are required for virus penetration of host cells and act as potent immuno-stimulants (Parekh et al., 1980). Within the *env* region, SU is thought to play a greater role in virulence than TM, based on results from studies of EIAV₁₇ virulence determinants (Payne et al., 2004). SU has a better ability to stimulate the production of antibodies than TM (Leroux et al., 1997). Based on studies of other lentiviruses, SU may interact with ELR1, the cellular receptor for EIAV (Leroux et al., 2004; Ball et al., 2005; Zhang et al., 2005). SU also evolves very rapidly during the course of infection and recurring disease (Hussain et al., 1987; Payne et al., 1989; Leroux et al., 1997). Ball et al., (2005) reported further evidence of the importance of SU as an EIAV virulence determinant when mouse pups injected with recombinant SU protein (SU₁₇) from virulent EIAV₁₇ developed dose-dependent diarrhea similar to that reported for SIV SU. Lim et al. (2005) also reported significant increases in IL-1 α , IL-1 β , IL-6, and TNF- α expression 0.5 to 1 hpi in EMDM infected with EIAV₁₇ as compared to those infected with EIAV₁₉. This project further extended these studies by examining the effects of SU protein on cytokine expression by using chimeric viruses with different combinations of SU and TM.

Two additional chimeric molecular clones, p17Nhe/19/wyoLTR and p19Nhe/17, producing viral stocks designated EIAV_{17SU} and EIAV_{17TM}, respectively, were developed to investigate the role of the *env* region in determining the virulence phenotype of EIAV₁₇ (Payne, et al., 2004). To determine if the SU region alone could modulate virulence, p17Nhe/19/wyoLTR contains only the SU region from the virulent parent

EIAV₁₇, while p19Nhe/17 contains only the TM/*Rev* region from EIAV₁₇ (Payne, et al., 2004). Although EIAV_{17SU} and EIAV_{17TM} have the same *gag*, *pol*, *tat*, *S2* and LTR sequences as EIAV₁₇, there is a significant difference in the construction of the two clones (Payne, et al., 2004). EIAV_{17SU} shares a segment of the SU region (from amino acids 147 to 444) with EIAV₁₇, while EIAV_{17TM} contains the SU region from EIAV₁₉ in addition to sharing some overlapping *rev* sequences and a portion of the TM region (from amino acids 20 to 417) with EIAV₁₇ (Payne, et al., 2004).

Although EIAV_{17SU} does not seem to be as virulent as EIAV₁₇ it does seem to show a greater virulence in ponies than EIAV_{17TM} (Payne et al., 2004). Shetland ponies infected with EIAV_{17SU} experience acute febrile episodes, unlike those infected with EIAV_{17TM}, which showed no change in normal body temperature 50 days post-infection (Payne, et al., 2004). Although the infecting dose of EIAV_{17SU} was up to 30-fold greater than that of the EIAV₁₇, the severity of resulting febrile episodes was reduced as compared to those previously observed in ponies infected EIAV₁₇ (Payne, et al., 2004). Therefore, the virulence phenotype of EIAV₁₇ cannot be attributed solely to the SU region, and that the addition of the TM/*Rev* sequences from EIAV₁₉ may result in attenuation of overall virulence (Payne, et al., 2004). Neither EIAV_{17SU} nor EIAV_{17TM} infection resulted acute febrile episodes requiring euthanasia of the ponies in this study, although ponies were infected with doses equivalent to or higher than the lethal dose of EIAV₁₇ (Payne, et al., 2004).

In order to expand on these previous studies that used ponies and EMDM, a set of QPCR assays for the detection of equine cytokines IL-1 α , IL-1 β , IL-6, IL-8 and TNF-

α were developed and validated. Primer and probe sets for IL-1 α , IL-1 β , IL-6, IL-8, TNF- α and 18S genes were designed using Applied Biosystems gene expression criteria (Allen et al., in press). First, available nucleotide sequences for each cytokine were compared using GenBank, and those with target regions that were fully conserved, free of base ambiguities, and not within alternatively spliced regions were selected and submitted to Applied Biosystem's Assays-by-DesignSM Service for development (Allen et al., in press).

After the primer and probe pairs were designed and developed, we determined the efficiency and reproducibility of the assays. The equine cytokine QPCR assays were evaluated using 5 criteria: 1) the inter-assay variation; 2) the intra-assay variation; 3) the variation in RNA extraction and cDNA synthesis; 4) the efficiency of the assays; and, 5) the amplification specificity of the assays. The amplification efficiency, intra-assay and inter-assay variation were determined using 10-fold dilutions of plasmid for each gene (Allen et al., in press). Under these conditions the amplification efficiencies of the primers and probes ranged from 99% to 101% (Table 2.2). The mean coefficient of variation (CV) across five sets of plasmid DNA for both intra-assay and inter-assay variation was 0.63% (range 0.2% to 1.8%) (Table 2.3). Amplification efficiency was also determined using 2-fold dilutions of cDNA, and under these conditions amplification efficiency ranged from 83% to 95% (Table 2.2). The specificity of amplification was confirmed by DNA sequencing of reaction products (Allen et al., in press). The QPCR assays were also evaluated using three sets of cDNA from EMDM stimulated for 1 h with LPS (Allen et al., in press). The general trend was the same for

all three samples with IL-1 α showing the greatest induction and IL-6 the lowest induction (Fig. 2.2). The range of cytokine induction was greater than has previously been reported with values ranging from 12-fold to 30,000-fold (Fig. 2.2). It was evident that the set of QPCR primers and probes were suitable for quantitation of expression equine cytokines in EMDM.

The set of QPCR assays were used to study the effects of EIAV₁₇, EIAV₁₉, EIAV_{17SU}, and EIAV_{17TM} on cytokine induction by EMDM. EMDM from three different horse donors was treated with one of the four clones or control media for 1 h. The cDNA prepared from the EMDM of the three horses was analyzed using QPCR in order to measure IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α expression. Overall impressions of the data were that cytokine expression levels varied greatly between horses. EMDM exposed to EIAV₁₇, EIAV_{17SU}, and EIAV_{17TM} showed an increase in induction levels in IL-1 α , IL-1 β , and TNF- α expression, but showed a decrease in expression of IL-6 and IL-8 (Table 3.1). Generally, EMDM exposed to EIAV₁₉ showed decrease in IL-1 α , IL-1 β , IL-6, IL-8, and TNF- α expression (Fig. 3.2, Fig. 3.3, and Fig. 3.4). Overall the expression of IL-1 α by EMDM ranged from 0.49- to 7.21-fold relative to CM (Fig. 3.2, Fig. 3.3, and Fig. 3.4). IL-1 β expression ranged from 0.36- to 7.86-fold relative to CM (Fig. 3.2, Fig. 3.3, and Fig. 3.4). TNF- α expression ranges from 0.27- to 4.44-fold relative to CM (Fig. 3.2, Fig. 3.3, and Fig. 3.4). Due to decreased expression in EMDM smaller ranges were seen for IL-8 (0.42- to 1.76-fold) and IL-6 (0.03- to 1.22-fold) (Fig. 3.2, Fig. 3.3, and Fig. 3.4). It appears that IL-6 and IL-8 expression in EMDM may have

been increased by CM treatment, thus lessening the effects of EIAV₁₇, EIAV_{17SU}, and EIAV_{17TM} treatment.

The first part of the hypothesis stated that a positive correlation exists between virulence phenotype and increased cytokine expression in macrophages, was supported by the data. The second part of the hypothesis that EIAV_{17SU} would show an increase in cytokine induction in EMDM as compared to EIAV_{17TM}, was neither proven nor disproven. The large horse-to-horse variation, combined with a small number of horses studied made it impossible to determine if the SU protein alone is responsible for modulating EIAV virulence and pathogenesis. Even with the small number of horses in this study, there was evidence of differences in cytokine expression between EMDM treated with the virulent EIAV₁₇ clone and those treated with the avirulent EIAV₁₉ clone. Perhaps a clearer picture of cytokine induction by EIAV_{17SU} will emerge from future studies that include a greater number of horses to offset the large horse-to-horse variation in cytokine induction seen in this study.

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APPENDIX A

FIGURES

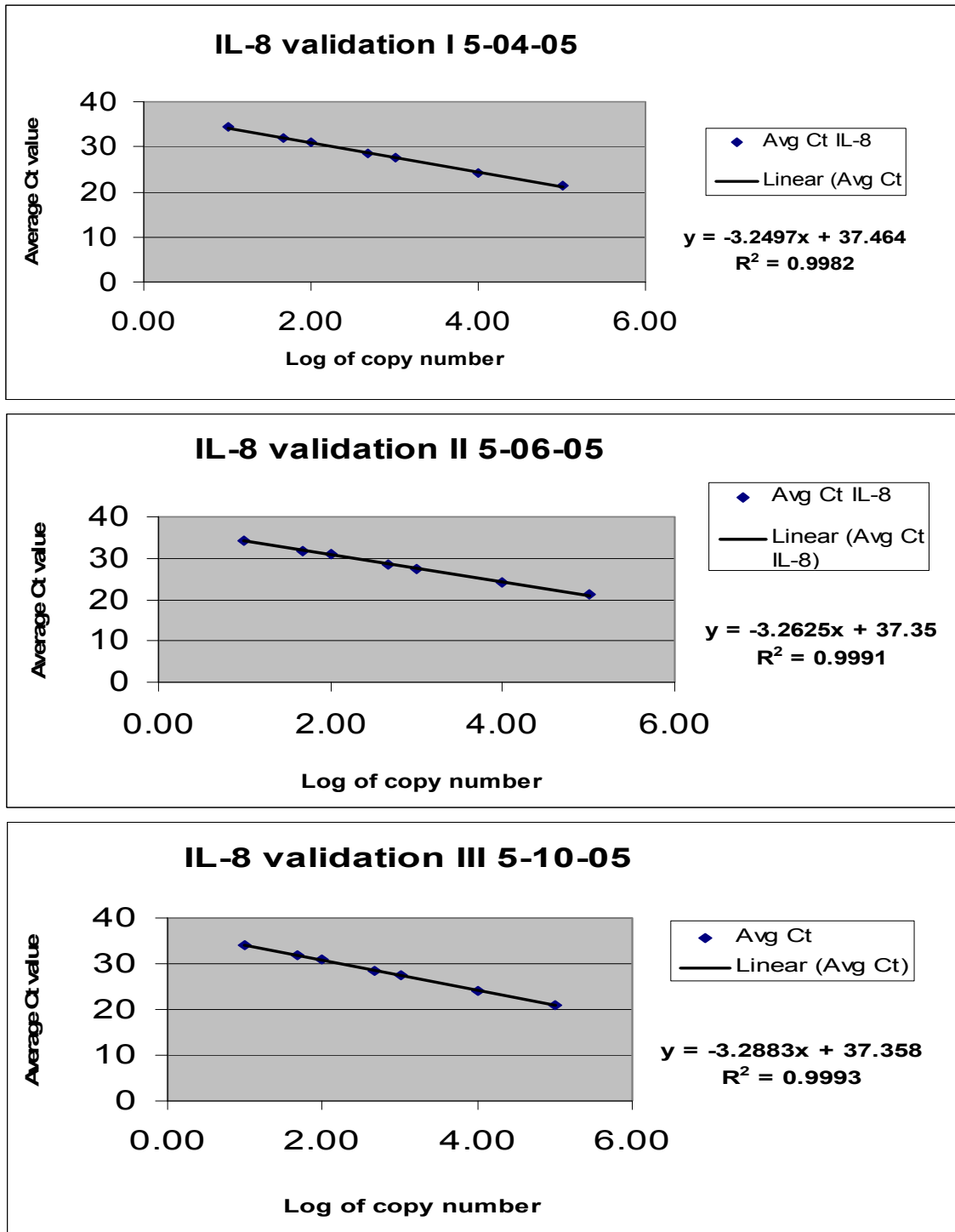


Fig. 1A. Standard curves used to determine inter-assay efficiency for IL-8 primers and probe. On the y-axis is the average threshold cycle value for IL-8 and on the x-axis is the log of the copy number for each IL-8 plasmid dilution. Three separate IL-8 QPCR assays were run on three separate days.

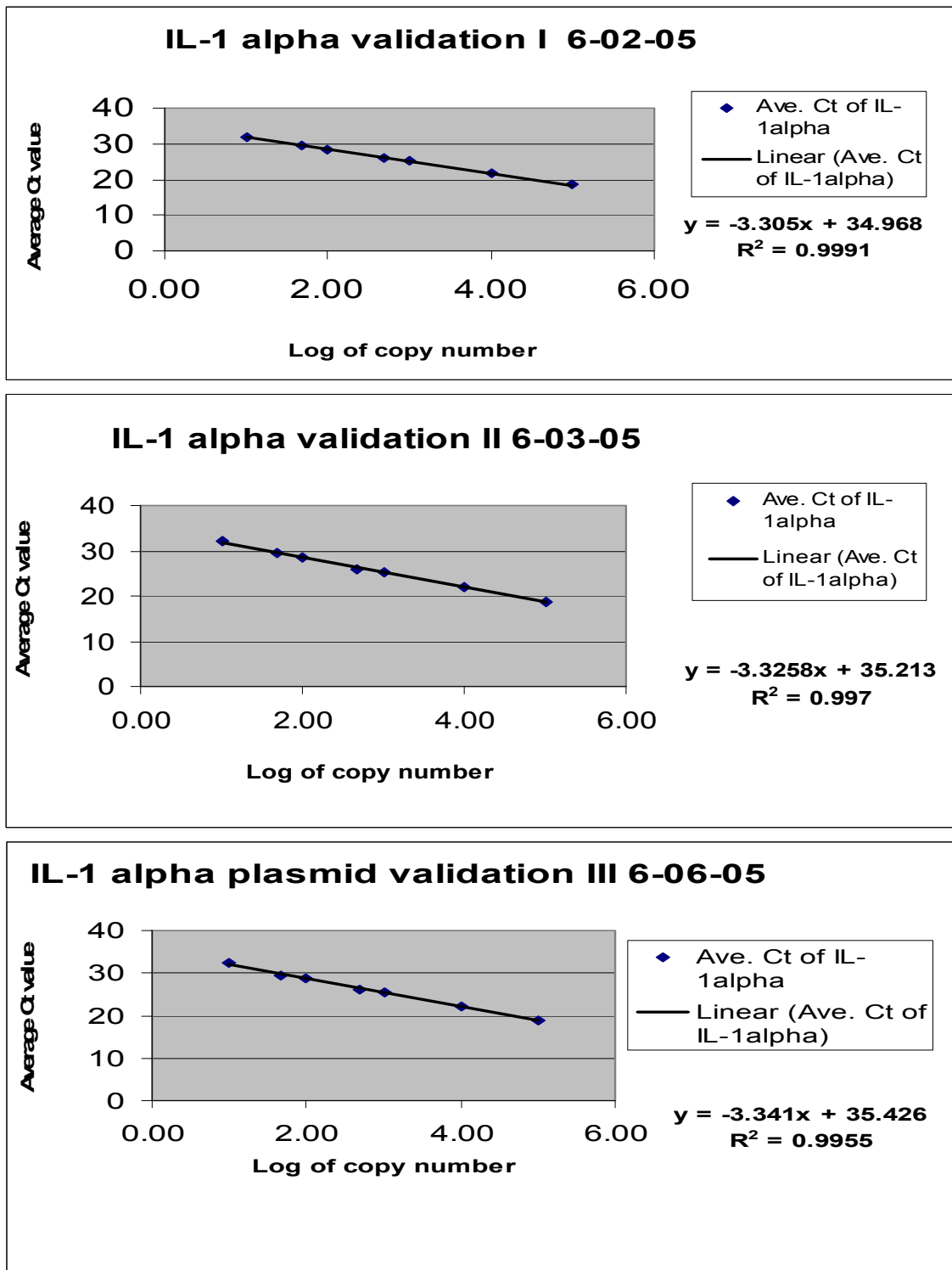


Fig. 2A. Standard curves used to determine inter-assay efficiency for IL-1 alpha primers and probe. The y-axis is the average threshold cycle value for IL-1alpha and the x-axis is the log of the copy number for each IL-1alpha plasmid dilution. Three separate IL-1alpha QPCR assays were run on three separate days.

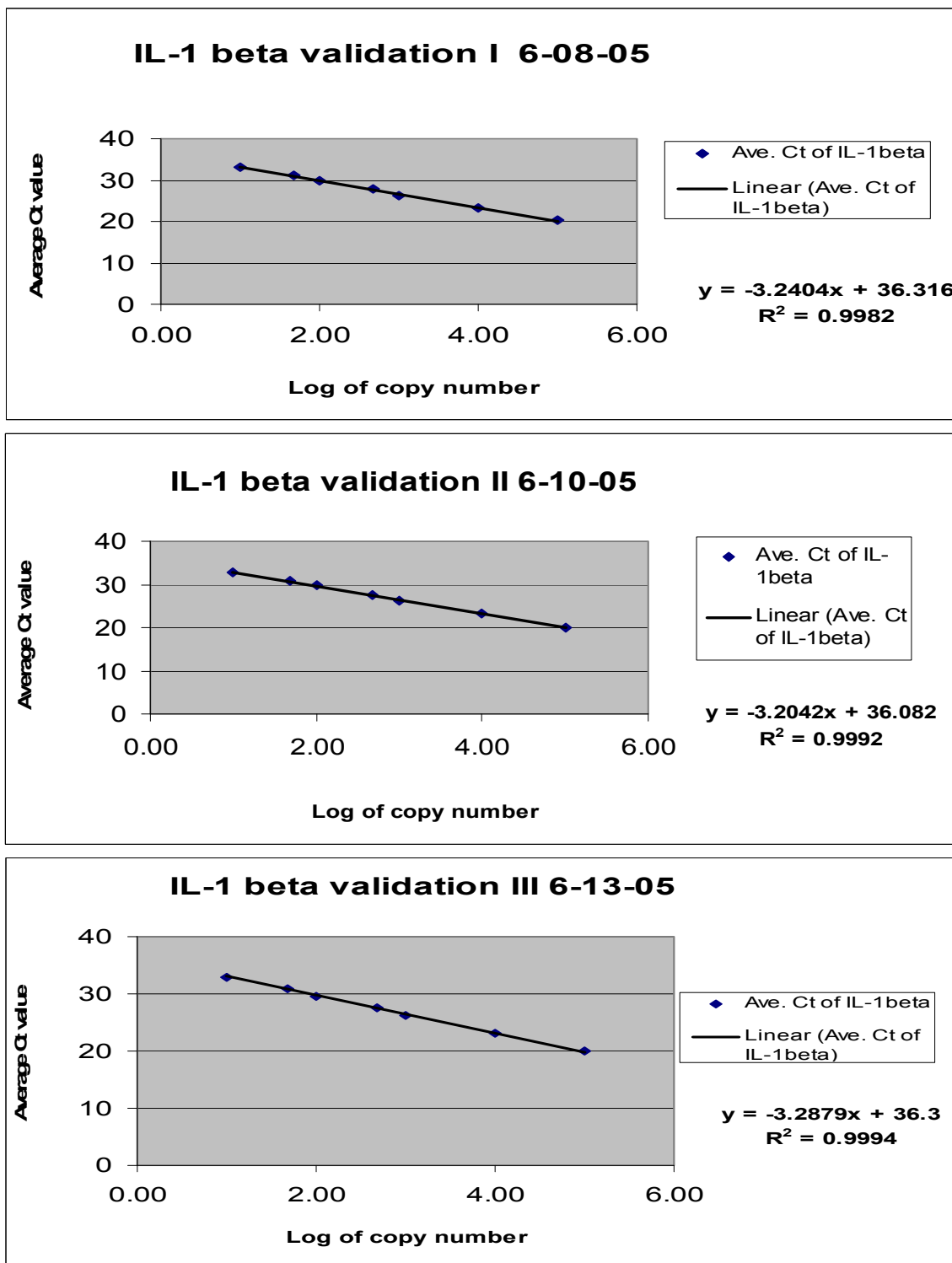


Fig. 3A. Standard curves used to determine inter-assay efficiency for IL-1 beta primers and probe. The y-axis is the average threshold cycle value for IL-1beta and the x-axis is the log of the copy number for each IL-1beta plasmid dilution. Three separate IL-1beta QPCR assays were run on three separate days.

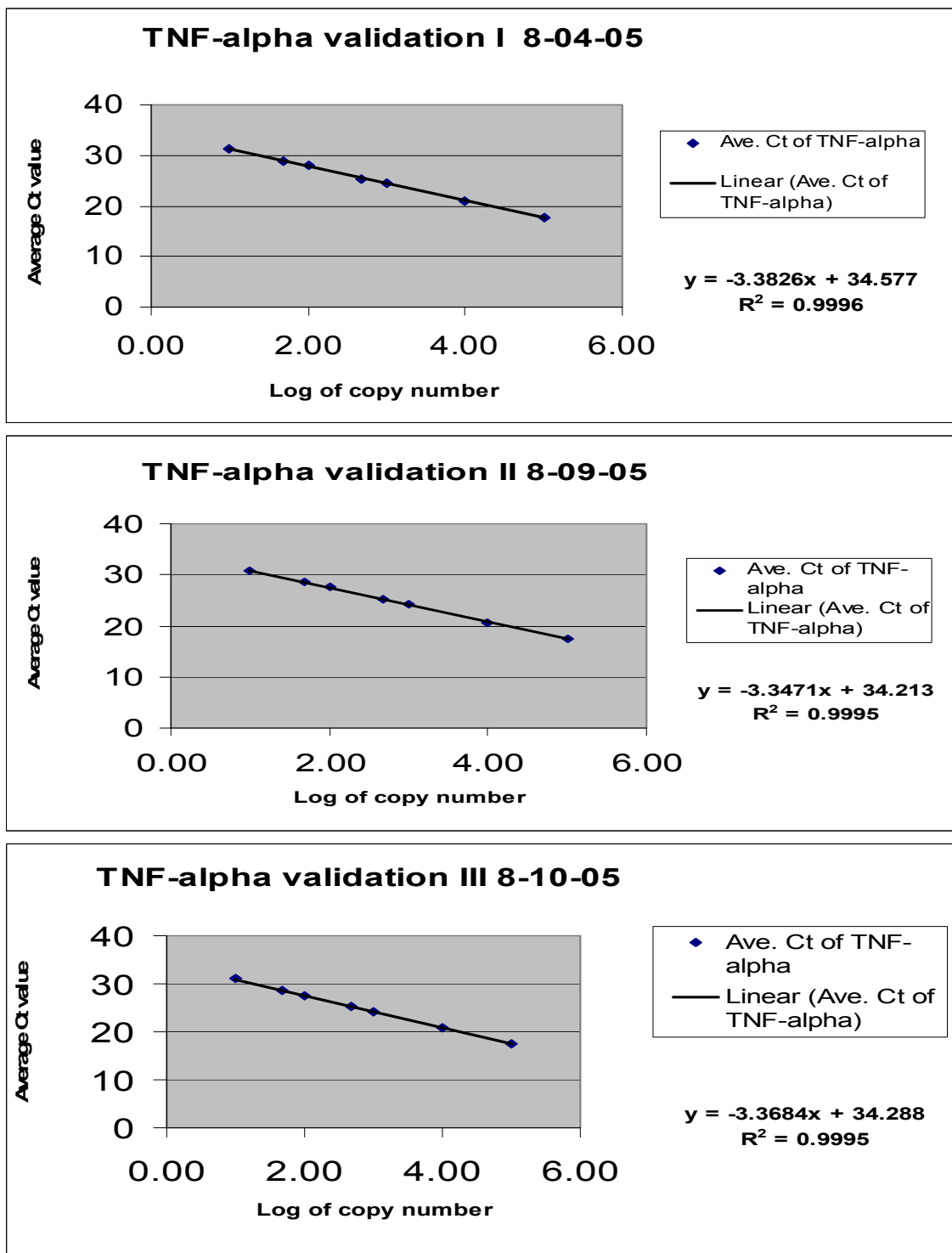


Fig. 4A. Standard curves used to determine inter-assay efficiency for TNF-alpha primers and probe. The y-axis is the average threshold cycle value for TNF-alpha and the x-axis is the log of the copy number for each TNF-alpha plasmid dilution. Three separate TNF-alpha QPCR assays were run on three separate days.

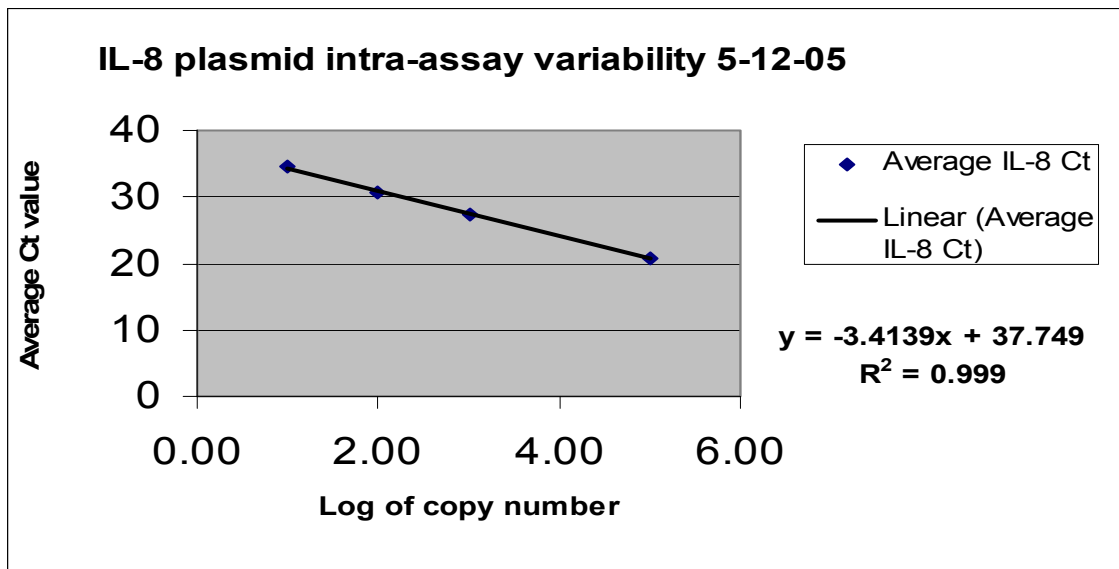


Fig. 5A. Standard curve used to determine intra-assay efficiency for IL-8 primers and probe. A single IL-8 QPCR assay was performed using 10 replicates of each of the following plasmid copy numbers 1×10^5 , 1×10^3 , 1×10^2 , and 1×10^1 .

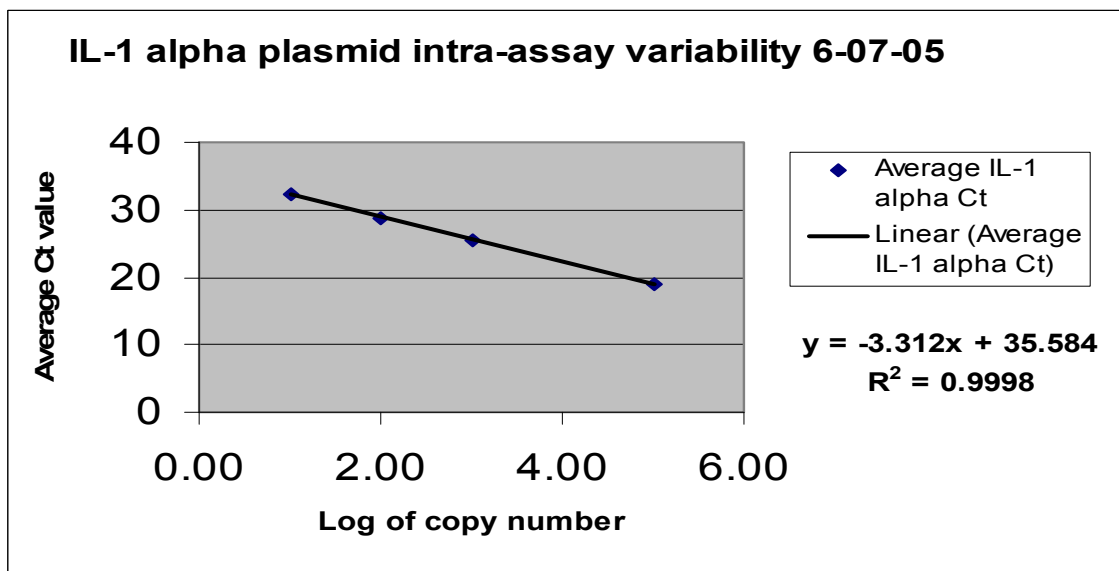


Fig. 6A. Standard curve used to determine intra-assay efficiency for IL-1 alpha primers and probe. A single IL-1 alpha QPCR assay was performed using 10 replicates of each of the following plasmid copy numbers 1×10^5 , 1×10^3 , 1×10^2 , and 1×10^1 .

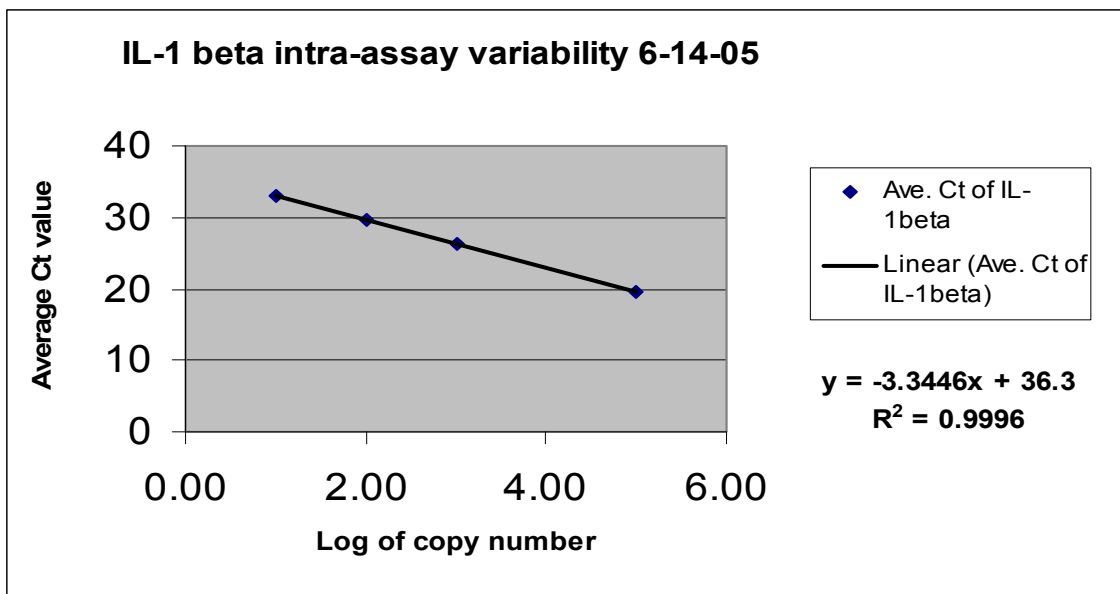


Fig. 7A. Standard curve used to determine intra-assay efficiency for IL-1 beta primers and probe. A single IL-1 beta QPCR assay was performed using 10 replicates of each of the following plasmid copy numbers 1×10^5 , 1×10^3 , 1×10^2 , and 1×10^1 .

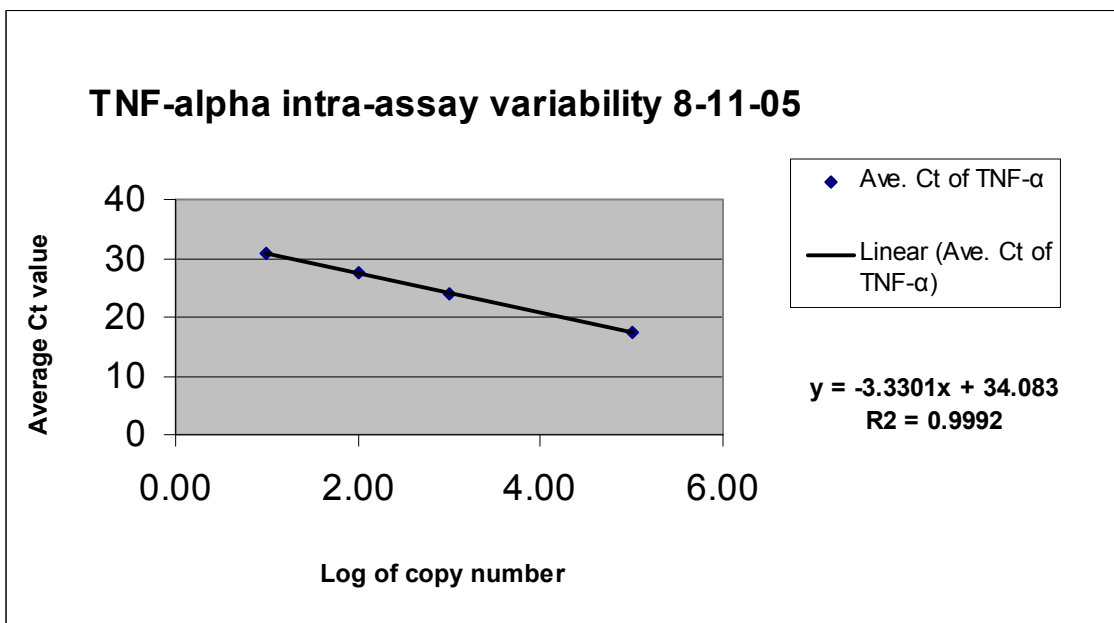


Fig. 8A. Standard curve used to determine intra-assay efficiency for TNF-alpha primers and probe. A single TNF-alpha QPCR assay was performed using 10 replicates of each of the following plasmid copy numbers 1×10^5 , 1×10^3 , 1×10^2 , and 1×10^1 .

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Board, V. V., Allen, C.A., Reeves, A., 1993. Ichthyofauna of a cypress–tupelo swamp. *Proceed. Ark. Acad. Sci.* 47,129.

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