EQUINE INNATE AND ADAPTIVE IMMUNITY TO VIRAL INFECTIONS

by

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B.S., Huazhong Agricultural University, 1991
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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Anatomy & Physiology
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Abstract

Activation of innate immunity through Toll-like receptor (TLR) signaling can also enhance antigen-specific adaptive immunity. TLR9 is an endosomal receptor for unmethylated bacterial and viral cytosine-phosphate-guanine DNA (CpG-DNA). West Nile virus (WNV) infection may result in meningitis and encephalitis in humans and horses, especially aged and immunocompromised individuals. Using flow cytometric analyses and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), we investigated equine cell-mediated immunity (CMI) to an inactivated West Nile virus vaccine in healthy yearling and adult horses. We also studied the potential of enhancing equine adaptive immunity to viruses and other pathogens by activation of innate immunity though TLR9 signaling pathway. We found vaccination with inactivated WNV vaccine induced strong WNV-specific T helper type 1 (Th1) and Th2 CMI with a Th1 bias, also effectively induced WNV-specific CTLs in yearling horses. In adult horses, the pre-existing Th1 CMI bias against WNV was enhanced following booster vaccination with inactivated WNV vaccine. Molecular characterization and flow cytometric analysis of TLR9 expression using a cross-reactive TLR9 mAb identified high constitutive expression of equine TLR9 in neutrophils (PMNs), $CD4^+$ and $CD8^+$ T cells and other leukocytes. Conservation of equine TLR9 and a high expression profile among leukocytes suggests that equine TLR9 is a frequent target for unmethylated CpG-DNA, an essential mechanism for the activation of innate immunity. Unmethylated CpG-DNA can significantly activate equine PMNs. It also induces expression of interferon (IFN)-α, IFN-β, IFN-γ, and interleukin (IL)-12p35 in PBMCs, as well as IFN-α and IFN-γ in monocyte-derived DCs. Enhanced expression of IFNs in immune cells by CpG-DNA is not only crucial for host viral clearance, but also important in mediating host immune responses due to IFNs' anti-inflammatory effects. Compared to the relatively weaker activation of equine innate immunity by inactivated WNV, the tested CpG-DNA species showed potential as vaccine adjuvants for enhancement of CTLs and Th1 CMI against intracellular pathogens, characterized by significant induction of type I IFNs and Th1-specific cytokines such as IL-12p35 and IFN-γ. These data provide a basis for further investigation of these CpG-DNA species as potentially effective vaccine adjuvants in horses.

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Dr. Elizabeth G. Davis

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Dedication

I sincerely dedicate this dissertation to my beloved wife Junfang, my lovely daughter Carol, my lovely son Jeffrey, and my parents. Their contribution to my PhD is far beyond my description. I could achieve little without their support.

CHAPTER 1 - Equine cell-mediated immune response to an inactivated West Nile virus vaccine

Abstract

West Nile virus (WNV) (*Flavivirus: Flaviviridae*) infection may result in meningitis and/or encephalitis in humans and horses, especially aged and immunosuppressed individuals. Immunization with West Nile Innovator®, an inactivated WNV vaccine, against WNV infection in equine with recommended doses has been very effective. However, except for humoral immunity, equine cell-mediated immunity (CMI) against WNV after vaccination with this inactivated WNV vaccine has not been unraveled. Compared to adult horses, juvenile horses may be defective in generating effective T helper (Th) type 1 immunity and susceptible to WNV infection. Utilizing flow cytometry, quantitative RT-PCR and carboxyfluorescein succinimidyl ester (CFSE)-based cell proliferation, we investigated CMI to vaccination with West Nile Innovator® in healthy yearling and adult horses. We found, in addition to strong WNV-specific humoral immune response as reported and in our study, the inactivated WNV vaccine can induce strong WNV-specific Th1 (IFN-γ secreting CD4 T cells) and Th2 (IL-4 secreting CD4 T cells) CMI with a Th1 bias, and also induce WNV-specific IFN-γ secreting CD8 T cells in yearling horses. In adult horses, the preexisted Th1 CMI bias against WNV can be enhanced and become more pronounced by booster vaccination with the inactivated WNV vaccine.

Keywords: Equine, West Nile virus, cell-mediated immunity, inactivated WNV vaccine

1. Introduction

WNV is a positive sense, approximately 11 kb single-stranded RNA arbovirus. Its genome encodes capsid (C), envelope (E), premembrane/membrane (prM/M), and 7 nonstructural (NS) proteins. This endemic arbovirus is mainly transmitted by *Culex* mosquitoes from birds, a major WNV reservoir. WNV infection can cause meningitis, encephalitis and encephalomyelitis, in which mortality can be as high as 10–15% in humans and 28-45% in horses (Brinton, 2002; Hayes et al., 2005; van der Meulen et al., 2005; Davis et al., 2006; Hayes and Gubler, 2006; Sejvar and Marfin, 2006; Kramer et al., 2008).

Studies of WNV disease using the murine model have been well-documented in recent years and clearly demonstrate that both innate immunity, including interferons (IFN-α, IFN-β and IFN-γ), γδT cells, and complement, and adaptive immunity, including cytotoxic T lymphocytes (CTLs) and Th1 cell-mediated immunity (CMI), and humoral immunity, are essential for the host to clear WNV, especially in the central nervous system (CNS) (Samuel and Diamond, 2006). In humans, the antigenic WNV viral epitopes for antibody (Ab) neutralization and presentation by major histocompatibility complex (MHC) classes I and II map to the C, M, E and all NS proteins (Sanchez et al., 2007; Lanteri et al., 2008; McMurtrey et al., 2008; Parsons et al., 2008), even though with varying degree of immune responses to epitopes among individuals. The optimal method for disease prevention against WNV is vaccination (Salazar et al., 2004; Schuler et al., 2004; Ward et al., 2004; Dauphin and Zientara, 2007; Gardner et al., 2007; Long et al., 2007; Chang et al., 2008), with either partial or all the viral antigens (whole attenuated or inactivated viruses) for induction of community immunity or herd immunity.

Currently there are four licensed WNV vaccines available for equines: West Nile-Innovator® and West Nile-Innovator DNA® (Fort Dodge Animal Health, Division of Wyeth, Fort Dodge, IA), Prevenile® (Intervet, De Soto, KS, equivalent to Chimerivax-WN®, Acambis, Cambridge, MA), and Recombitek® equine WNV vaccine (Merial Limited, Athens, GA) (Meeusen et al., 2007; Kramer et al., 2008). West Nile-Innovator® is formalin-inactivated WNV vaccine with the adjuvant Metastim® (Fort Dodge Animal Health). West Nile-Innovator DNA® was the first licensed DNA vaccine (Davis et al., 2001). Prevenile® is a live attenuated chimeric WNV/YFV (yellow fever virus) vaccine without adjuvant (Long et al., 2007; Seino et al., 2007). Recombitek® is a recombinant canarypox virus vaccine with adjuvant (Minke et al., 2004; Siger et al., 2004; El Garch et al., 2008). The last three vaccines all encode the E and preM proteins as antigens.

West Nile-Innovator® was the first licensed WNV vaccine and thus mostly tested in small and large scale trials for safety and vaccine efficacy (Ng et al., 2003; Vest et al., 2004; Epp et al., 2005). This killed WNV vaccine has demonstrated an efficacy of about 95% against WNV disease (Ng et al., 2003; Epp et al., 2005) in small (20 horses) and large scale (875 horses) studies. Although elevated levels of serum neutralizing antibodies due to vaccination likely play an important role in clearing WNV in horses, whether vaccination with this inactivated WNV can induce CMI against WNV has not been reported, especially in yearling horses. Here we evaluated the CMI against WNV in healthy yearling and adult horses after vaccination with West Nile-Innovator® and found that this killed WNV vaccine stimulated robust antigen-specific Th1 CMI (IFN-γ secreting CD4 T cells) as well as IFN-γ secreting CD8 T cells against WNV in yearling horses. Such a Th1 CMI immune response was more pronounced in adult horses.

2. Materials and Methods

2.1. Animals, vaccination schedule and blood sampling

All animal protocols were approved by the Institutional Animal Care and Use Committee at Kansas State University. There were two treatment groups of healthy yearling horses: saline placebo and vaccination (West Nile Innovator®), with 6 horses per group. Yearling horses were vaccinated three times with an interval of three weeks. Peripheral blood samples (heparinzed) were collected by jugular venipuncture for evaluating immune responses to vaccination at baseline (0 day), 1, 2, 3, 4, 5, 8, 10, 12, 16, 20, 24, 28 and 32 weeks. Serum samples from these yearling horses were tested for WNV infection by IgM capture ELISA and plaque reduction neutralization test (PRNT) at the National Veterinary Services Laboratories (NVSL, Ames, Iowa) as well as ELISA of all Ab classes and IgG subclasses specific to the WNV preM and E proteins in our lab to monitor humoral immunity and natural exposure to WNV throughout the experiment.

Six healthy adult horses (13 to 21 years old) that were previously vaccinated against WNV once or twice annually were utilized to characterize the CMI against WNV in adults after

vaccination with the killed WNV vaccine. Blood samples were collected before the initial annual booster vaccination against WNV, then 1 and 2 weeks after vaccination.

2.2. Cell Culture

Equine peripheral blood mononuclear cells (PBMCs) were isolated by density gradient and cultured as reported (Zhang et al., 2008). Cultured PBMCs were treated with or without purified WNV antigen (pre-membrane + envelope proteins, 20 μg/ml) for 3-5 days. Then cells were treated with Golgiplug^{TM} (protein transport inhibitor Brefeldin A, BD Sciences, San Jose, CA) for 12 hrs before harvest for cytokine staining.

Some PMBCs were stained with carboxyfluorescein succinimidyl ester (CFSE) for tracking WNV-specific cell proliferation with the following modified method (Lyons and Parish, 1994; Hodgkin et al., 1996). Briefly, cells (2 x 10^6/ ml) were stained with 1μM CFSE at room temperature in the dark for 15 min. The staining was terminated by adding 4-5 ml of equine serum and RPMI 1640 (Hyclone, Logan, UT) to bring the volume up to 25 ml in a 50 ml tube. CFSE-stained PBMCs were centrifuged for 10 min at 400 g at room temperature. After decanting the supernatant, the cells were resuspended for culture. After culturing with or without purified WNV antigens for 5 days, cells were then counted and harvested for surface immunophenotyping by flow cytometry.

2.3. Abs and flow cytometry

Primary mouse anti-equine CD4 (HB61A), CD8 (HT14A) and MHC classes I (H58A) and II (H42A) mAbs were purchased from VMRD (Pullman, WA). Secondary goat anti-mouse Abs, APC-conjugated, were from Invitrogen (Carlsbad, CA). The cross-reactive mouse anti-bovine IFN-γ (CC302, AbD Serotec, Raleigh, NC) (Pedersen et al., 2002; Paillot et al., 2005) and IL-4 (CC303, AbD Serotec) (Pedersen et al., 2002) mAbs were from AbD Serotec and their isotype control mAbs (clone MOPC-21, FITC or PE conjugated) from BD Sciences Pharmingen. The cross-reactivity of mouse anti-bovine IL-4 with equine IL-4 was confirmed with horse

PBMCs treated with PHA (5 ug/1x 10^6 cells/ml) or PMA plus ionomycin as reported (Wagner et al., 2006). Similar results were obtained (data not shown).

For all animals, complete blood counts (CBC) were performed and leukocytes were immuno-phenotyped on CD4, CD8, MHC classes I and II by flow cytometry to ensure these animals had no immune deficiency (Davis et al., 2002). For investigation of CMI, cultured PBMCs were first surface-stained with CD4 and CD8 mAbs, then fixed and permeablized for intracellular IFN-γ and IL-4 mAb staining, following the protocol with the FIX & PERM kit (Invitrogen). For cell proliferation, CFSE stained cells were surface-stained with Abs against CD4, CD8, MHC class I and II for flow cytometric analysis as reported (Zhang et al., 2008). Only viable cells (based on propidium iodide staining of separate samples) were gated for flow cytometric analysis.

Flow cytometric two-color dot plot analysis was performed using CellQuest (BD Sciences) or Winlist 6.0 software (Verity Software House, Topsham, ME).

2.4. Quantitative RT-PCR

Quantitative RT-PCR was performed on DNase I-digested total RNA samples from individual animals using the QuantiFast SYBR Green RT-PCR Kit (Qiagen Inc, Valencia, CA) in a Bio-Rad iCycler iQ Real Time PCR System (Bio-Rad, Hercules, CA).

Quantitative RT-PCR for 18S rRNA (as the internal control), IFN-γ and IL-4 was performed in 15-ul reactions with 15 ng total RNA and gene-specific primers (Table 1.1). The specificity of every primer pair was confirmed by sequencing amplicon if unique product was amplified based on electrophoresis in 1-2% agarose gel, as well as melting-curve analysis in real time RT-PCR. Negative controls without total RNA were included for each run of real time RT-PCR. The PCR amplification efficiency of each reaction was estimated by linear regression, in which 3 to 6 points covering the Ct value were utilized based on the highest correlation coefficiency (Ramakers et al., 2003). The averaged PCR efficiency for each gene was utilized for computing relative gene expression (Pfaffl, 2001; Cikos et al., 2007). IFN-γ and IL-4 data were normalized to 18S rRNA.

2.5. Data Analyses

Prior to vaccination, all yearling horses except one were negative of WNV IgM capture ELISA and PRNT as well as no difference between the two groups in WNV-specific IgM, IgA, IgG(T), IgGa, IgGb and IgGc, and no recalled WNV-specific CMI was detected in these animals. The PRNT for WNV for one of the vaccinated horses was found positive (titer >1:10), so this horse was considered as a positive control and excluded for all data analysis. After 10 weeks post primary vaccination, three of the yearling control horses were found PRNT positive for WNV, thus no data after this point were shown even though random natural exposure to WNV infection did not affect the difference in immunity to WNV between vaccinated and control (placebo) animals.

Based upon the flow cytometric data from two-color contour dot plots in either CellQuest (BD Sciences) or Winlist 6.0 (Verity Software House), the WNV antigen specific immune response was computed as the difference between WNV stimulation and culture media. One way analysis of variance or Kruskal-Wallis one way analysis of variance on ranks in SigmaStat 3.5 was performed for all data analyses. Paired *t*-test in SigmaStat 3.5 was performed for analyzing the relative IFN-γ and IL-4 expression in PBMCs. $p < 0.05$ is considered statistically significant.

3. Results

3.1. WNV-specific CMI

To investigate the CMI elicited by vaccination with killed WNV vaccine in horses, purified PBMCs were cultured with and without WNV antigen stimulation. We measured IFN-γ (Fig. 1.1. A & B) and IL-4 production by CD4 T cells, and IFN-γ production by CD8 T cells by flow cytometry. Then we evaluated the WNV-specific IFN-γ and IL-4 production by CD4 T cells, and IFN-γ production by CD8 T cells, respectively as the difference between WNV antigen-stimulation and media alone.

3.1.1. WNV-specific Th1 CMI

IFN-γ production by CD4 T cells is critical for the clearance of viral and other intracellular pathogens. Thus, the induction of antigen-specific Th1 CMI by vaccination is a crucial parameter for evaluating vaccine efficacy (Kaech et al., 2002; Lambert et al., 2005; Seder et al., 2008). Fig. 1.1A & B demonstrated our flow cytometric assay of WNV-specific IFN-γ production by CD4 T cells. Compared to control horses, which showed little WNV-specific IFN-γ secreting CD4 T cells, vaccinated yearling horses consistently showed significantly higher frequency of WNV-specific IFN-γ secreting CD4 T cells since 14 days after primary vaccination. Such a Th1 CMI was enhanced by the first and second booster vaccinations. The dynamic trend in Th1 CMI response to vaccination during a series of time points varied considerably among animals (Fig. 1.1 C). Ten weeks after primary vaccination, this Th1 CMI decreased but maintained (data not shown).

3.1.2. WNV-specific Th2 CMI

The induction of antigen-specific IL-4 producing CD4 T cells is often observed after vaccination (Lambert et al., 2005), especially in youngsters because youngsters, particularly neonates, are limited in Th1 CMI and their immature CMI is Th2 biased (Adkins et al., 2004; Breathnach et al., 2006). Likewise for the killed WNV vaccine, control horses showed no recalled Th2 CMI response to vaccination, but vaccinated yearling horses consistently showed significantly higher frequency of WNV-specific IL-4 secreting CD4 T cells since 14 days after primary vaccination. Such a Th2 CMI was also enhanced by the first and second booster vaccinations (Fig. 1.2). Ten weeks after primary vaccination, this Th2 CMI decreased but was maintained significantly (data not shown).

3.1.3. WNV-specific CD8 T cells

Antigen-specific CTL (mainly of IFN-γ secreting CD8 T cells) activity also is critical for the clearance of viral and other intracellular pathogens (Kaech et al., 2002; Lambert et al., 2005;

Seder et al., 2008). Compared to control horses, vaccinated yearling horses showed significantly higher frequency of recalled WNV-specific CD8 T cells at 7 and 14 days after the first booster vaccination, but appeared not enhanced by the second booster vaccination (Fig. 1.3). Ten weeks after primary vaccination, this CTL activity still maintained (data not shown). The frequency of WNV-specific IFN-γ secreting CD8 T cells was much lower than that of WNV-specific IFN-γ secreting CD4 T cells. Similar results also were found in adult horses after booster vaccination with killed WNV vaccine (data not shown).

3.1.4. WNV-specific Th1 vs Th2 CMI

Th2 CMI might function antagonistically against Th1 CMI for young hosts to balance their immature adaptive immunity and prevent possible deleterious impacts from exuberant IFN-γ producing CD4 T cells. The balance between Th1 and Th2 CMI might somehow indicate the efficacy of vaccination. We chose vaccinated healthy adult horses as positive controls for comparison of Th1 and Th2 CMI because their host immune responses to vaccination should be protective after long term immune selection through either vaccination and/or natural infection. Healthy yearling horses showed a Th1-biased CMI against WNV even though the Th2 CMI was also strong (Fig. 1.4A). Compared to the relative magnitudes of Th1 and Th2 CMI to killed WNV vaccine in yearlings, adult horses demonstrated a dominant Th1 CMI against WNV at baseline and boosted responses 1 and 2 weeks after the booster vaccination (Fig. 1.4B).

3.1.5. Quantitative RT-PCR of IFN-γ and IL-4 expression

To further confirm the WNV-specific induction of IFN-γ and IL-4 by T cells, using quantitative RT-PCR, we analyzed the relative expression of IFN-γ and IL-4 by PBMCs with and without WNV antigen stimulation at a few relatively comparable time points in yearling and adult horses: baseline, 2 weeks after the first booster vaccination for yearling horses, and 2 weeks after the booster vaccination for adult horses. The adult horses demonstrated dramatic fold increase in WNV-specific IFN-γ production over IL-4 after WNV antigen stimulation in vitro, the yearling horses showed a balanced WNV-specific IFN-γ and IL-4 production (Fig. 1.5A). After *in vitro* WNV antigen stimulation, the ratio of IFN-γ to IL-4 expression by PBMCs from

yearling horses was about 40 fold lower in average than that from adult horses (Fig. 1.5B). These data supported the flow cytometric data of Th1 and Th2 CMI against WNV.

3.2. WNV-specific cell proliferation

CFSE-based cell proliferation is a widely-applied effective technique to evaluate antigen-specific CMI by flow cytometry, in which CFSE is measured by FL1 height, the green channel detector (Lyons and Parish, 1994; Lyons, 2000). If immunological reagents available, multiple-color flow cytometric analysis can be carried out to simultaneously characterize multiparameters of antigen-specific cell proliferation and its regulation (Krupnick et al., 2001; Hilchey and Bernstein, 2007). In our study, we mainly investigated the WNV-specific proliferation of CD4 and CD8 T cells as well as their activation status based on MHC class II expression by flow cytometry. In panels A and B of Fig. 1.6, we demonstrated the flow cytometric assay of WNV-specific CD4 T cell proliferation from a typical vaccinated yearling horse. Compared to control horses, vaccinated yearling horses consistently showed a significantly higher frequency of WNV-specific CD4 T cells proliferation 7 days after primary vaccination. Such a proliferating CMI, including both Th1 and Th2 CMI, was enhanced by the first and second booster vaccinations (Fig. 1.6C). Also, compared to controls, vaccinated yearling horses showed a significantly higher percentage of WNV-specific CD8 T cell proliferation 14 days after the first booster vaccination. Such a proliferating CD8 T cells was little enhanced by the second booster vaccinations (Fig. 1.6D), and vaccinated yearling horses consistently showed significantly higher frequency of MHC class II cell proliferation since 7 days after primary vaccination (Fig. 1.6E). The WNV-specific MHC class II cell proliferation appeared highly synchronous with the WNV-specific proliferation of CD4 and CD8 T cells and was also enhanced by the first and second booster vaccinations because MHC class II expression on CD4 and CD8 T cells may be associated with all T cell activation (Lunn et al., 1993). Similar results were observed in adult horses regarding the WNV-specific proliferation of CD4 and CD8 T cells as well as their activation status based on MHC class II expression (data not shown).

4. Discussion

Our study showed that vaccination with an inactivated WNV vaccine induced WNV antigen-specific, protective Th1 CMI (Fig. 1.1) as well as humoral immunity (antigen-specific IgA, IgG(T), IgGa, IgGb and IgGc, data not shown) in yearling horses and such adaptive CMI was enhanced by booster vaccination. Even though Th2 CMI against WNV was also induced by vaccination, Th1 CMI against WNV was dominant in yearling horses and shown to be more pronounced in adult horses. WNV-specific CD8 T cell response was also induced consistently and significantly after the first booster vaccination in yearling horses and this immune response was more pronounced in adult horses due to booster vaccination. Two of the vaccinated yearling horses (H6 and H9 in Fig. 1.1, 1.2 & 1.6) showed relative weaker CMI, either Th1 or Th2, than the other three vaccinated yearling horses, however, they were still able to acquire equivalent WNV-specific Ab levels of IgA and all IgG subclasses as of other vaccinated ones (data not shown).

We demonstrated that the CFSE-based WNV-specific CD4 and CD8 T cell proliferation as well as their corresponding activation status in terms of surface expression MHC class II in yearling horses after vaccination (Fig. 1.6). Similar results were also observed in vaccinated adult horses (data not shown).

Because antigen-specific immune responses in healthy adults should be protective through immune selection by the host under pathogenic challenge during growth and development, thus their preexisted Th1 CMI is enhanced by booster vaccination and should be induced quickly for protection if natural WNV infection occurs. Whether such an effective WNV-specific Th1 CMI in healthy adult horses is due to natural WNV infection or vaccination does not compromise, but only confirms that WNV-specific protective Th1 CMI can be induced in yearling horses by vaccination with the killed WNV vaccine.

In the recall assay of CMI, we used incomplete WNV antigens (just preM and E proteins) for *in vitro* PBMC stimulation (same antigens for ELISA). These two antigens may not stimulate maximal recall cellular response as measured from PBMCs. Two of the vaccinated horses that consistently showed low Th1 CMI response to WNV preM and E proteins may have stronger Th1 CMI response to the C and NS proteins. Flow cytometric analysis was very sensitive and showed a significant difference in CMI between control and vaccinated yearling horses (Fig. 1.1,

1.2 & 1.6). These data were indirectly confirmed by our quantitative RT-PCR assay of IFN-γ and IL-4 expression in T cells after stimulation with WNV antigens (Fig. 1.5).

An issue for the killed WNV vaccine is the typical relatively weaker CD8 T cells recall response in comparison with Th1 CMI (Lambert et al., 2005), as shown in yearling horses (Fig. 1.1, 1.3 & 1.6) as well as in adult horses (data not shown). Also, the function of high level of WNV-specific IL-4 secreted by CD4 T cells in yearling horses after vaccination with the killed WNV vaccine is unknown. Such IL-4 production could contribute to the production of non-opsonizing Abs, or the regulation of IFN-γ secreting CD4 T cells (Kaech et al., 2002).

Many reports have shown the efficacy of killed WNV vaccine with recommended doses (Ng et al., 2003; Schuler et al., 2004; Ward et al., 2004; Davidson et al., 2005; Epp et al., 2005; Seino et al., 2007). One of these reports suggested that the immunogenicity of killed WNV vaccine might be weak in some horses based on the low titers (≤1:10) of WNV plaque reduction neutralization test (PRNT) (Davidson et al., 2005) at 4 to 6 weeks after vaccination in comparison with naturally infected horses at 4 to 6 weeks after disease onset. Since there was no challenge study for these vaccinated horses, such lower serum Ab titers against WNV in some vaccinated horses can also be interpreted that these horses cleared WNV more effectively and quickly, possibly with their more efficient innate immunity and/or WNV-specific adaptive immunity (CMI and Abs).

Prevenile® and Recombitek® vaccines have also been showing good efficacy against WNV infection in horses (Minke et al., 2004; Siger et al., 2004; Long et al., 2007; Seino et al., 2007; El Garch et al., 2008). However, horses vaccinated once with Prevenile® or twice with Recombiteck® only showed less than 1/44 or 1/8 of WNV neutralizing Ab level in horses vaccinated twice with West Nile-Innvator® before WNV challenge (Seino et al., 2007). This explains why Prevenile® or Chimerivax-WN® showed less vaccine efficacy against WNV infection than what is claimed (95%) (Long et al., 2007). The low neutralizing Ab level induced by Recombitek® is possibly due to strong host humoral response to the canarypox virus vector, thus little booster effect was found in WNV-specific Th1 CMI and humoral immunity (El Garch et al., 2008). In comparison to Prevenile® and Recombitek® vaccines, WNV antigens in killed WNV vaccine are more heterogenous, in which all the NS proteins are included as targets of CMI and humoral immunity (Sanchez et al., 2007; McMurtrey et al., 2008; Parsons et al., 2008). This may explain why West Nile Innovator® is better at the induction of WNV-specific humoral

immunity than Prevenile® and Recombitek® vaccines (Balasuriya et al., 2006; Seino et al., 2007) as well as showing clearer consistent induction of Th1 CMI and CTLs (Fig. 1.1, 1.3 & 1.6), even though this vaccine elicited slightly more clinical signs like swelling in the injection sites than the chimeric WNV vaccines (Ng et al., 2003; Seino et al., 2007). However, it is impossible for Prevenile® and Recombitek® to induce immune responses to the WNV NS proteins in animals simply because these two vaccines do not encode these NS proteins. Compared to chimeric live attenuated WNV vaccines, vaccines of killed WNV or purified WNV antigens may have more room for improvement of WNV-specific CTL and humoral responses with the help of effective adjuvants and vaccination strategy that can mimic natural viral infections (Zinkernagel and Hengartner, 2001; Davidson et al., 2005; Johansen et al., 2008; Lanteri et al., 2008), yet more researches still need to be done.

In conclusion, in addition to strong WNV-specific humoral immune response as reported and in our study, the killed WNV vaccine can induce strong WNV-specific Th1 and Th2 CMI with a Th1 bias, and also induce WNV-specific CTLs in yearling horses. In adult horses, the preexisted Th1 CMI bias against WNV is more pronounced and can be enhanced by booster vaccination with the killed WNV vaccine.

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References

Adkins, B., Leclerc, C., Marshall-Clarke, S., 2004. Neonatal adaptive immunity comes of age. Nat Rev Immunol 4, 553-564.

Balasuriya, U.B., Shi, P.Y., Wong, S.J., Demarest, V.L., Gardner, I.A., Hullinger, P.J., Ferraro, G.L., Boone, J.D., De Cino, C.L., Glaser, A.L., Renshaw, R.W., Ledizet, M., Koski, R.A., MacLachlan, N.J., 2006. Detection of antibodies to West Nile virus in equine sera using microsphere immunoassay. J. Vet. Diagn. Invest. 18, 392-395.

Breathnach, C.C., Sturgill-Wright, T., Stiltner, J.L., Adams, A.A., Lunn, D.P., Horohov, D.W., 2006. Foals are interferon gamma-deficient at birth. Vet. Immunol. Immunopathol. 112, 199-209.

Brinton, M.A., 2002. The molecular biology of West Nile Virus: a new invader of the western hemisphere. Annu. Rev. Microbiol. 56, 371-402.

Chang, D.C., Liu, W.J., Anraku, I., Clark, D.C., Pollitt, C.C., Suhrbier, A., Hall, R.A., Khromykh, A.A., 2008. Single-round infectious particles enhance immunogenicity of a DNA vaccine against West Nile virus. Nat. Biotechnol. 26, 571-577.

Cikos, S., Bukovska, A., Koppel, J., 2007. Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. BMC Mol Biol 8, 113.

Dauphin, G., Zientara, S., 2007. West Nile virus: recent trends in diagnosis and vaccine development. Vaccine 25, 5563-5576.

Davidson, A.H., Traub-Dargatz, J.L., Rodeheaver, R.M., Ostlund, E.N., Pedersen, D.D., Moorhead, R.G., Stricklin, J.B., Dewell, R.D., Roach, S.D., Long, R.E., Albers, S.J., Callan, R.J., Salman, M.D., 2005. Immunologic responses to West Nile virus in vaccinated and clinically affected horses. J. Am. Vet. Med. Assoc. 226, 240-245.

Davis, B.S., Chang, G.J., Cropp, B., Roehrig, J.T., Martin, D.A., Mitchell, C.J., Bowen, R., Bunning, M.L., 2001. West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. J. Virol. 75, 4040-4047.

Davis, E.G., Wilkerson, M.J., Rush, B.R., 2002. Flow cytometry: clinical applications in equine medicine. J. Vet. Intern. Med. 16, 404-410.

Davis, L.E., DeBiasi, R., Goade, D.E., Haaland, K.Y., Harrington, J.A., Harnar, J.B., Pergam, S.A., King, M.K., DeMasters, B.K., Tyler, K.L., 2006. West Nile virus neuroinvasive disease. Ann. Neurol. 60, 286-300.

El Garch, H., Minke, J.M., Rehder, J., Richard, S., Edlund Toulemonde, C., Dinic, S., Andreoni, C., Audonnet, J.C., Nordgren, R., Juillard, V., 2008. A West Nile virus (WNV)

recombinant canarypox virus vaccine elicits WNV-specific neutralizing antibodies and cell-mediated immune responses in the horse. Vet. Immunol. Immunopathol. 123, 230-239.

Epp, T., Waldner, C., West, K., Leighton, F., Townsend, H., 2005. Efficacy of vaccination for West Nile virus in Saskatchewan horses. AAEP Proc. 51, 180-182.

Gardner, I.A., Wong, S.J., Ferraro, G.L., Balasuriya, U.B., Hullinger, P.J., Wilson, W.D., Shi, P.Y., MacLachlan, N.J., 2007. Incidence and effects of West Nile virus infection in vaccinated and unvaccinated horses in California. Vet. Res. 38, 109-116.

Hayes, E.B., Gubler, D.J., 2006. West Nile virus: epidemiology and clinical features of an emerging epidemic in the United States. Annu. Rev. Med. 57, 181-194.

Hayes, E.B., Sejvar, J.J., Zaki, S.R., Lanciotti, R.S., Bode, A.V., Campbell, G.L., 2005. Virology, pathology, and clinical manifestations of West Nile virus disease. Emerg. Infect. Dis. 11, 1174-1179.

Hilchey, S.P., Bernstein, S.H., 2007. Use of CFSE to monitor ex vivo regulatory T-cell suppression of CD4+ and CD8+ T-cell proliferation within unseparated mononuclear cells from malignant and non-malignant human lymph node biopsies. Immunol. Invest. 36, 629-648.

Hodgkin, P.D., Lee, J.H., Lyons, A.B., 1996. B cell differentiation and isotype switching is related to division cycle number. J. Exp. Med. 184, 277-281.

Johansen, P., Storni, T., Rettig, L., Qiu, Z., Der-Sarkissian, A., Smith, K.A., Manolova, V., Lang, K.S., Senti, G., Mullhaupt, B., Gerlach, T., Speck, R.F., Bot, A., Kundig, T.M., 2008. Antigen kinetics determines immune reactivity. Proc. Natl. Acad. Sci. U. S. A. 105, 5189-5194.

Kaech, S.M., Wherry, E.J., Ahmed, R., 2002. Effector and memory T-cell differentiation: implications for vaccine development. Nat Rev Immunol 2, 251-262.

Kramer, L.D., Styer, L.M., Ebel, G.D., 2008. A global perspective on the epidemiology of West Nile virus. Annu. Rev. Entomol. 53, 61-81.

Krupnick, A.S., Kreisel, D., Szeto, W.Y., Popma, S.H., Amin, K.M., Moore, J.S., Rosengard, B.R., 2001. Multiparameter flow cytometric approach for simultaneous evaluation of T lymphocyte-endothelial cell interactions. Cytometry 46, 271-280.

Lambert, P.H., Liu, M., Siegrist, C.A., 2005. Can successful vaccines teach us how to induce efficient protective immune responses? Nat. Med. 11, S54-62.

Lanteri, M.C., Heitman, J.W., Owen, R.E., Busch, T., Gefter, N., Kiely, N., Kamel, H.T., Tobler, L.H., Busch, M.P., Norris, P.J., 2008. Comprehensive analysis of west nile virus-specific T cell responses in humans. J. Infect. Dis. 197, 1296-1306.

Long, M.T., Gibbs, E.P., Mellencamp, M.W., Bowen, R.A., Seino, K.K., Zhang, S., Beachboard, S.E., Humphrey, P.P., 2007. Efficacy, duration, and onset of immunogenicity of a West Nile virus vaccine, live Flavivirus chimera, in horses with a clinical disease challenge model. Equine Vet. J. 39, 491-497.

Lunn, D.P., Holmes, M.A., Duffus, W.P., 1993. Equine T-lymphocyte MHC II expression: variation with age and subset. Vet. Immunol. Immunopathol. 35, 225-238.

Lyons, A.B., 2000. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. J. Immunol. Methods 243, 147-154.

Lyons, A.B., Parish, C.R., 1994. Determination of lymphocyte division by flow cytometry. J. Immunol. Methods 171, 131-137.

McMurtrey, C.P., Lelic, A., Piazza, P., Chakrabarti, A.K., Yablonsky, E.J., Wahl, A., Bardet, W., Eckerd, A., Cook, R.L., Hess, R., Buchli, R., Loeb, M., Rinaldo, C.R., Bramson, J., Hildebrand, W.H., 2008. Epitope discovery in West Nile virus infection: Identification and immune recognition of viral epitopes. Proc. Natl. Acad. Sci. U. S. A. 105, 2981-2986.

Meeusen, E.N., Walker, J., Peters, A., Pastoret, P.P., Jungersen, G., 2007. Current status of veterinary vaccines. Clin. Microbiol. Rev. 20, 489-510, table of contents.

Minke, J.M., Siger, L., Karaca, K., Austgen, L., Gordy, P., Bowen, R., Renshaw, R.W., Loosmore, S., Audonnet, J.C., Nordgren, B., 2004. Recombinant canarypoxvirus vaccine carrying the prM/E genes of West Nile virus protects horses against a West Nile virus-mosquito challenge. Arch. Virol. Suppl., 221-230.

Ng, T., Hathaway, D., Jennings, N., Champ, D., Chiang, Y.W., Chu, H.J., 2003. Equine vaccine for West Nile virus. Dev. Biol. (Basel) 114, 221-227.

Paillot, R., Daly, J.M., Juillard, V., Minke, J.M., Hannant, D., Kydd, J.H., 2005. Equine interferon gamma synthesis in lymphocytes after in vivo infection and in vitro stimulation with EHV-1. Vaccine 23, 4541-4551.

Parsons, R., Lelic, A., Hayes, L., Carter, A., Marshall, L., Evelegh, C., Drebot, M., Andonova, M., McMurtrey, C., Hildebrand, W., Loeb, M.B., Bramson, J.L., 2008. The Memory T Cell Response to West Nile Virus in Symptomatic Humans following Natural Infection Is Not Influenced by Age and Is Dominated by a Restricted Set of CD8+ T Cell Epitopes. J. Immunol. 181, 1563-1572.

Pedersen, L.G., Castelruiz, Y., Jacobsen, S., Aasted, B., 2002. Identification of monoclonal antibodies that cross-react with cytokines from different animal species. Vet. Immunol. Immunopathol. 88, 111-122.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29, e45.

Ramakers, C., Ruijter, J.M., Deprez, R.H., Moorman, A.F., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62-66.

Salazar, P., Traub-Dargatz, J.L., Morley, P.S., Wilmot, D.D., Steffen, D.J., Cunningham, W.E., Salman, M.D., 2004. Outcome of equids with clinical signs of West Nile virus infection and factors associated with death. J. Am. Vet. Med. Assoc. 225, 267-274.

Samuel, M.A., Diamond, M.S., 2006. Pathogenesis of West Nile Virus infection: a balance between virulence, innate and adaptive immunity, and viral evasion. J. Virol. 80, 9349-9360.

Sanchez, M.D., Pierson, T.C., Degrace, M.M., Mattei, L.M., Hanna, S.L., Del Piero, F., Doms, R.W., 2007. The neutralizing antibody response against West Nile virus in naturally infected horses. Virology 359, 336-348.

Schuler, L.A., Khaitsa, M.L., Dyer, N.W., Stoltenow, C.L., 2004. Evaluation of an outbreak of West Nile virus infection in horses: 569 cases (2002). J. Am. Vet. Med. Assoc. 225, 1084-1089.

Seder, R.A., Darrah, P.A., Roederer, M., 2008. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol 8, 247-258.

Seino, K.K., Long, M.T., Gibbs, E.P., Bowen, R.A., Beachboard, S.E., Humphrey, P.P., Dixon, M.A., Bourgeois, M.A., 2007. Comparative efficacies of three commercially available vaccines against West Nile Virus (WNV) in a short-duration challenge trial involving an equine WNV encephalitis model. Clin Vaccine Immunol 14, 1465-1471.

Sejvar, J.J., Marfin, A.A., 2006. Manifestations of West Nile neuroinvasive disease. Rev. Med. Virol. 16, 209-224.

Siger, L., Bowen, R.A., Karaca, K., Murray, M.J., Gordy, P.W., Loosmore, S.M., Audonnet, J.C., Nordgren, R.M., Minke, J.M., 2004. Assessment of the efficacy of a single dose of a recombinant vaccine against West Nile virus in response to natural challenge with West Nile virus-infected mosquitoes in horses. Am. J. Vet. Res. 65, 1459-1462.

van der Meulen, K.M., Pensaert, M.B., Nauwynck, H.J., 2005. West Nile virus in the vertebrate world. Arch. Virol. 150, 637-657.

Vest, D.J., Cohen, N.D., Berezowski, C.J., Morehead, J.P., Blodgett, G.P., Blanchard, T.L., 2004. Evaluation of administration of West Nile virus vaccine to pregnant broodmares. J. Am. Vet. Med. Assoc. 225, 1894-1897.

Wagner, B., Hillegas, J.M., Antczak, D.F., 2006. A monoclonal antibody to equine interleukin-4. Vet. Immunol. Immunopathol. 110, 363-367.

Ward, M.P., Levy, M., Thacker, H.L., Ash, M., Norman, S.K., Moore, G.E., Webb, P.W., 2004. Investigation of an outbreak of encephalomyelitis caused by West Nile virus in 136 horses. J. Am. Vet. Med. Assoc. 225, 84-89.

Zhang, Y.W., Davis, E.G., Blecha, F., Wilkerson, M.J., 2008. Molecular cloning and characterization of equine Toll-like receptor 9. Vet. Immunol. Immunopathol. 34, 209-219.

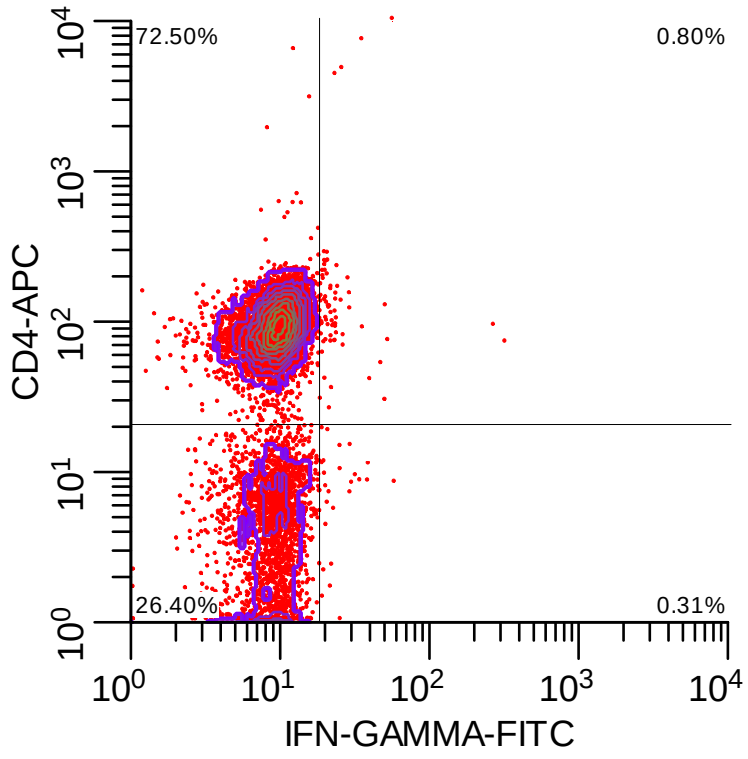
Zinkernagel, R.M., Hengartner, H., 2001. Regulation of the immune response by antigen. Science 293, 251-253.

Table 1.1. Primers for real time RT-PCR

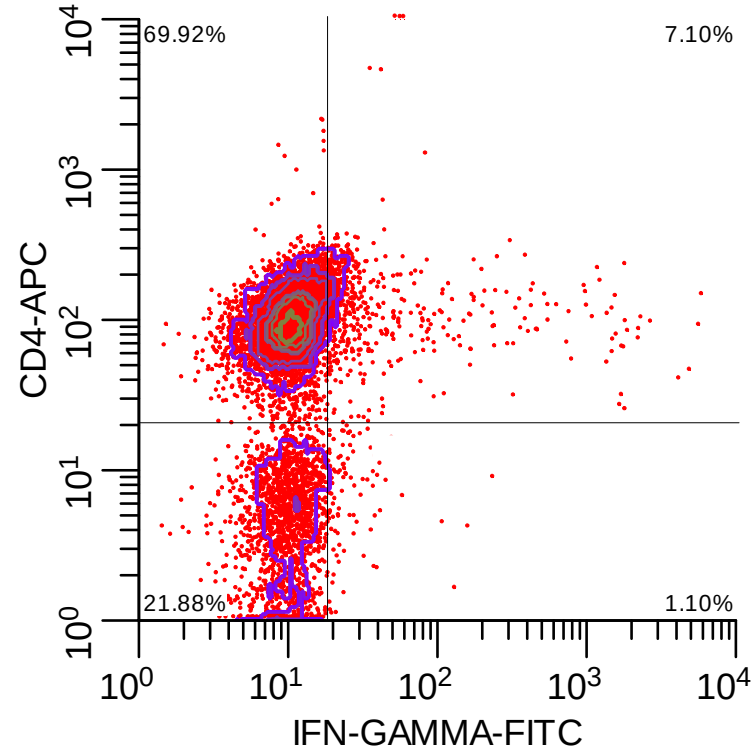
Gene	Primer	Sequence (5' → 3')	Amplicon Length (bp)	Genbank accession No.
18S rRNA	Forward	ATGCGGCGGCGTTATTCC	204	AJ311673
	Reverse	GCTATCAATCTGTCAATCCTGTCC		
IFN-γ	Forward	GCCAAATCGTCTCCTTCTACTTC	260	D28520
	Reverse	CTGACTCCTCTTCCGCTTCC		
IL-4	Forward	GAACAACCTCACAGATGGAAAG	238	AF305617
	Reverse	GCTCTTCTTGGCTTCATTCAC		

Figures

(A) Media



(B) WNV stimulated



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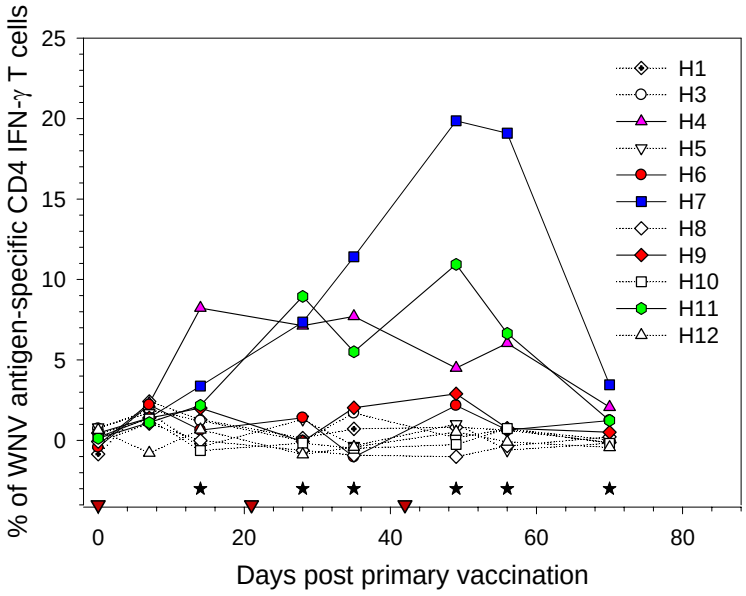


Fig. 1.1. Vaccination with an inactivated WNV vaccine elicited effective Th1 CMI in yearling horses. Flow cytometric assay of WNV-specific Th1 CMI from a typical vaccinated horse was shown in panels A and B, all samples were gated on the viable cells that were identified by propidium iodide staining. (A) The IFN-γ secreting CD4 T cells cultured without

purified WNV antigens. (B) The IFN-γ secreting CD4 T cells cultured with purified WNV antigens (20 ug preM+ E proteins/ml). Brefeldin A was added to cells and incubated for 12 hr after all cells were cultured for 4 days. Then cells were harvested and stained for surface antigens and intracellular cytokines. (C) WNV-specific IFN-γ secretion by CD4 T cells in control (H1, H3, H5, H8, H10 and H12, with unfilled and uncolored symbols), vaccinated horses (H4, H6, H7, H9 and H11, with filled and colored symbols). The line/scatter plot instead of box and whisker plot showed the dynamic trends in host immune response to vaccination during a series of time points, but with high variation among animals. The stars indicated a statistically significant difference between control and vaccination animals. The inverted red-filled triangles on the X axis indicated the days for vaccination.

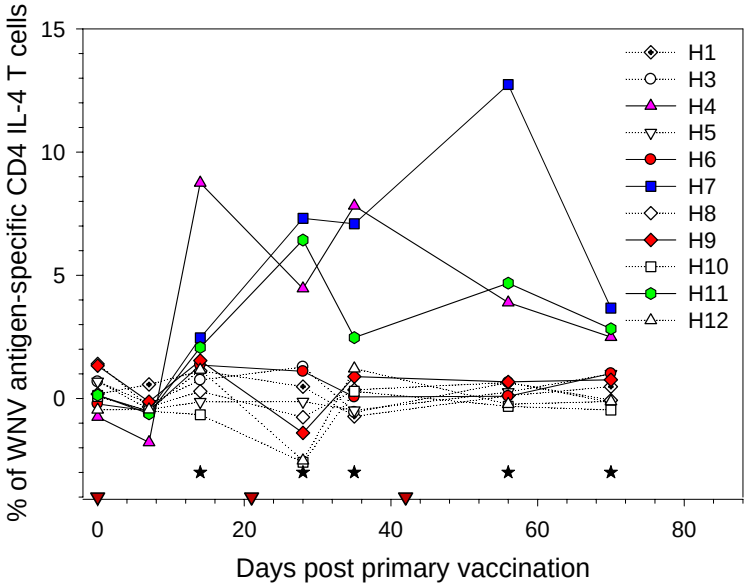


Fig. 1.2. Vaccination with an inactivated WNV vaccine elicited WNV-specific Th2 CMI in yearling horses. Samples were from exactly the same treatments, stained the same way as in Fig. 1.1, except stained for IL-4 instead of IFN-γ. Figure legends were the same as in Fig. 1.1.

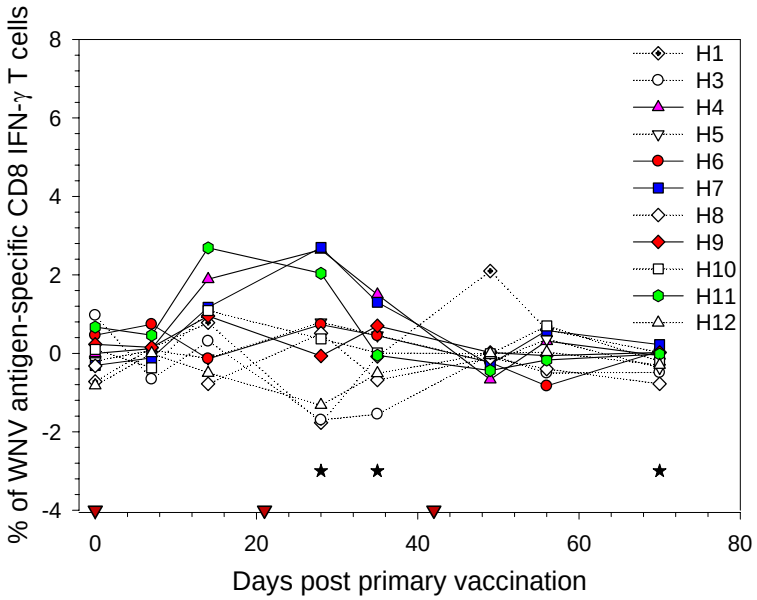
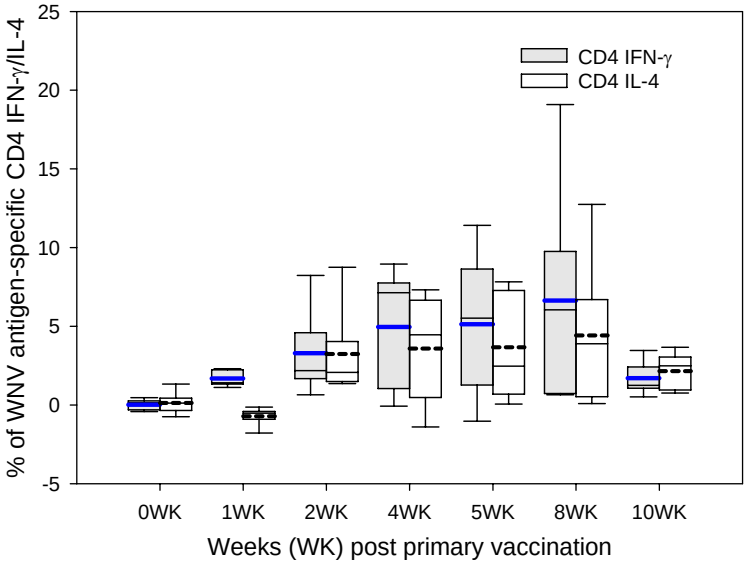


Fig. 1.3. Vaccination with an inactivated WNV vaccine elicited WNV-specific CTLs in yearling horses. Samples were from exactly the same treatments, stained the same way as in Fig. 1.1, except stained for CD8 instead of CD4. Figure legends were the same as in Fig. 1.1.

(A)



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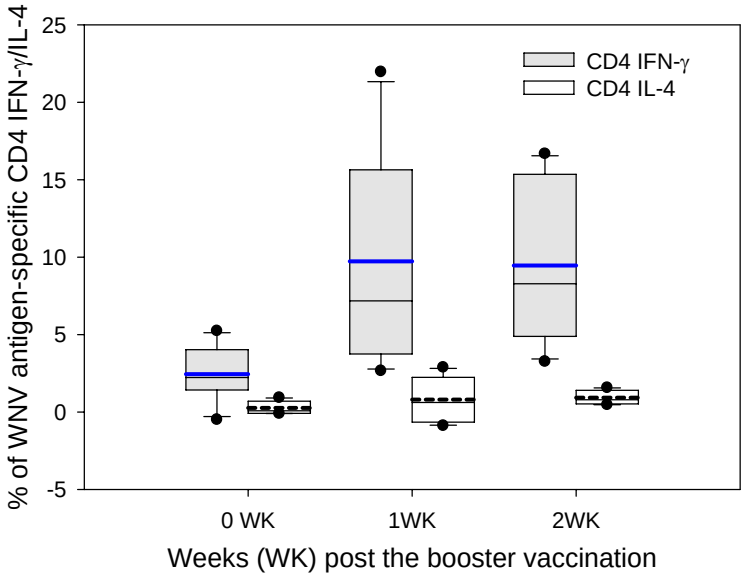
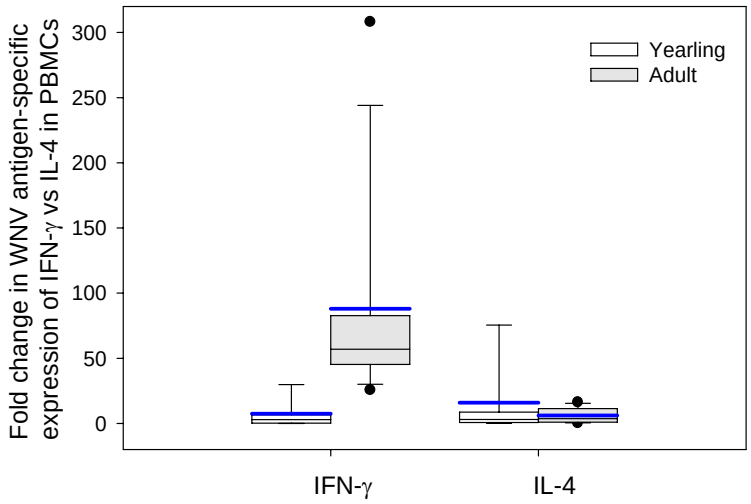


Fig. 1.4. Comparison of WNV-specific Th1 and Th2 CMI (flow cytometric data) in vaccinated healthy yearling and adult horses. (A) Yearling horses (n=5) have both Th1 and Th2 cell-mediated immune responses (with a Th1 bias) to WNV vaccination. The data were from Fig. 1.1 and 2. (B) Adult horses (n=6) have much stronger Th1-biased cell-mediated immune response to WNV. These horses were previous vaccinated against WNV. These data were obtained 1 and 2 weeks after the last booster immunization. Their PBMCs, except being cultured for 3 days instead of 4 days, were treated and stained exactly the same way as shown in Fig. 1.1 and 1.2. The difference between IFN-γ and IL-4 is significant, $p<0.002$. Since most of the data were not normally distributed, box-and-whisker plots in Sigmaplot 10 (Systat Software, Inc., San Jose, CA) were applied to show the data (same for Fig. 1.5). The solid thick blue or dashed thick black bar is for the mean, the thin black bar for the median.

(A)



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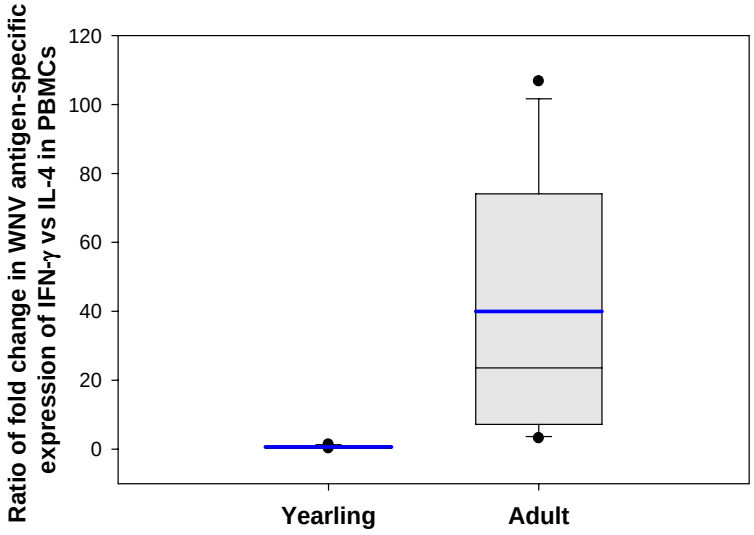
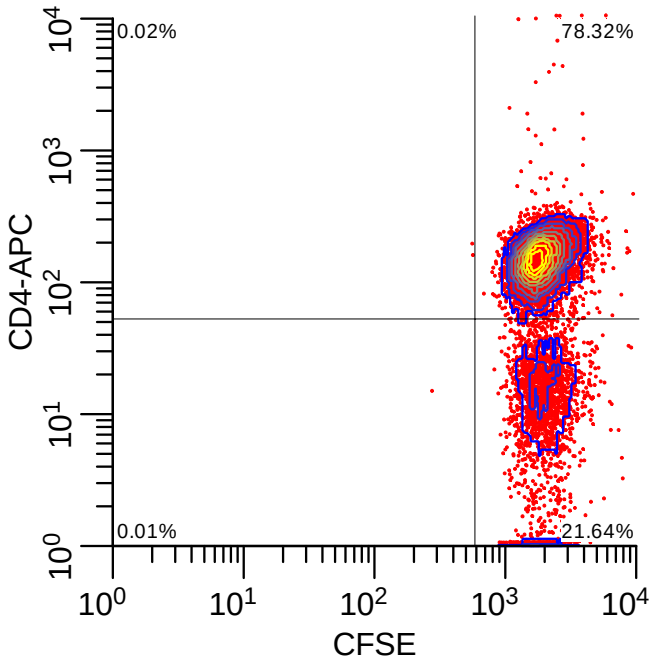


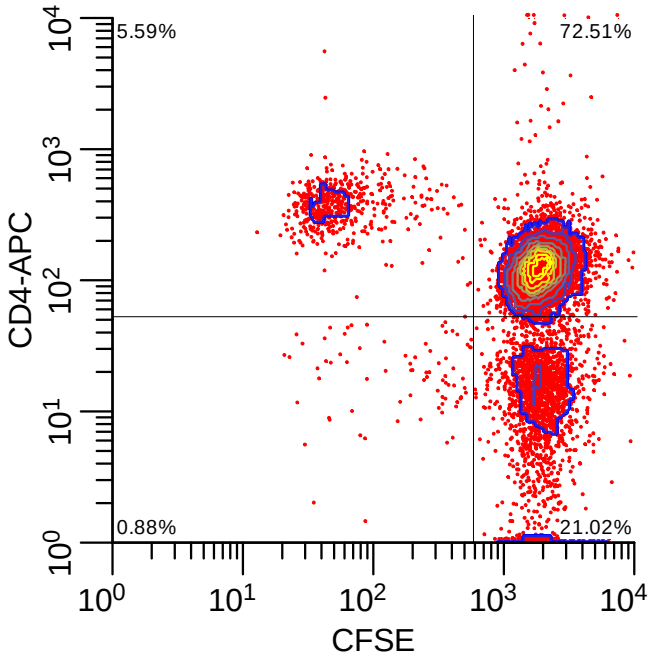
Fig. 1.5. Comparison of relative fold changes in expression of WNV-specific IFN-γ and IL-4 by PBMCs from vaccinated healthy yearling and adult horses. Quantitative one-step RT-PCR was performed to quantify the relative expression of IFN-γ and IL-4 with 18S rRNA as the internal control. (A) The expression of Th1 cytokine IFN- γ and Th2 cytokine IL-4 in PBMCs from vaccinated yearling (n=5) and adult horses (n=6) after WNV antigen stimulation in vitro. The solid thick blue bar is for the mean, the thin black bar for the median. The samples collected at 2 weeks after the 1st booster vaccination for yearling horses, and at 2 weeks after the booster vaccination for adults were compared. (B) Change in ratio of IFN- γ to IL-4 expression by

PBMCs in yearling and adult horses after WNV antigen stimulation in vitro. The difference between yearling and adult horses is statistically significant (p value = 0.02).

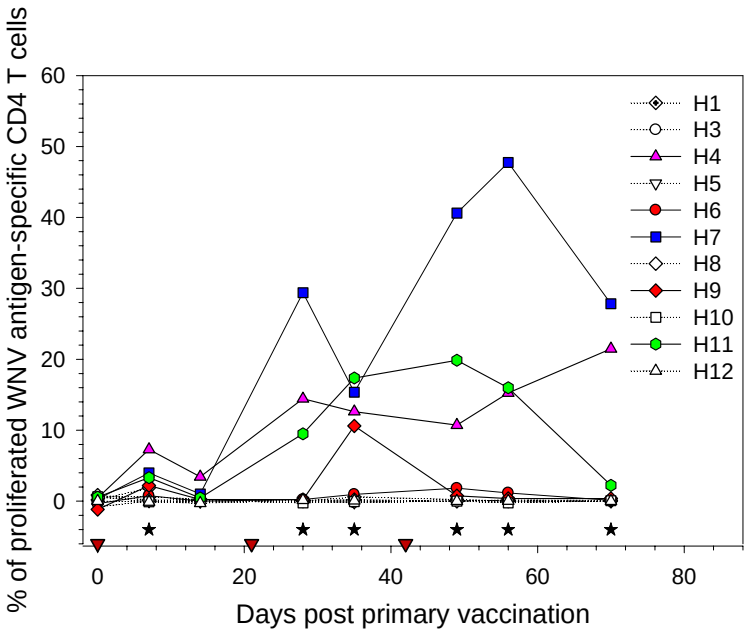
(A) Media



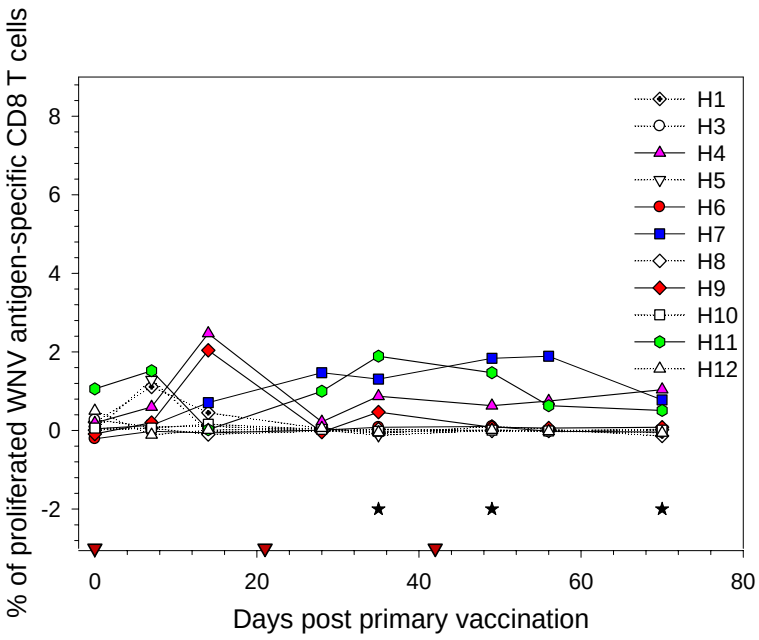
(B) WNV stimulated



(C)


% of proliferated WNV antigen-specific CD4 T cells
60
50
40
30
20
10
0
0
20
40
60
80
Days post primary vaccination
H1
H3
H4
H5
H6
H7
H8
H9
H10
H11
H12

(D)


% of proliferated WNV antigen-specific CD8 T cells
8
6
4
2
0
-2
0
20
40
60
80
Days post primary vaccination
H1
H3
H4
H5
H6
H7
H8
H9
H10
H11
H12

(E)

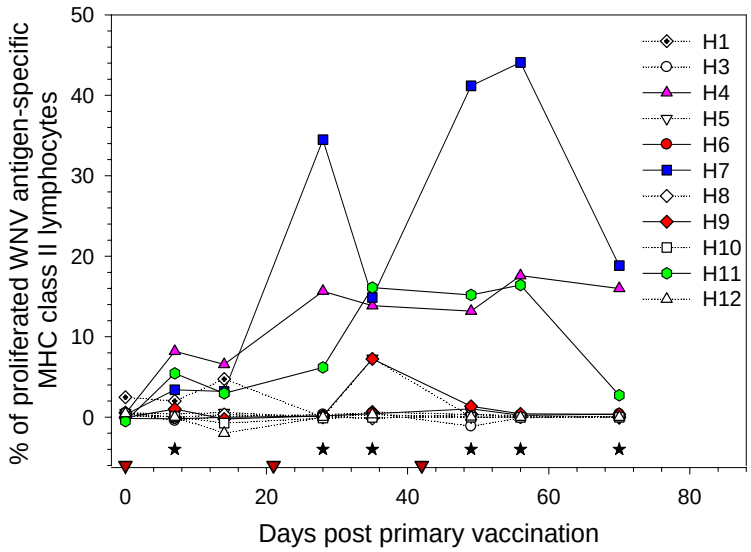


Fig. 1.6. Vaccination with an inactivated WNV vaccine elicited strong WNV-specific CD4 T cell proliferation in yearling horses. Flow cytometric assay of WNV-specific CD4 T cell proliferation from a typical vaccinated horse was shown in panels A and B, all cells were CFSE-stained and cultured for 5-6 days. (A) CFSE-stained PBMCs were not stimulated purified WNV antigens. (B) CFSE-stained PBMCs were stimulated with purified WNV antigens (20 ug preM+ E proteins/ml). Cells were only surface-stained. For panels C, D and E, the figure legends were the same as in Fig. 1.1. (C) The frequency or percentage of WNV-specific CD4 T cells proliferation for control and vaccinated horses. (D) The percentage of WNV-specific proliferating CTLs for control and vaccinated horses. (E) The percentage of WNV-specific MHC class II+ cell proliferation for control and vaccinated horses.

CHAPTER 2 - Molecular cloning and characterization of equine Toll-like receptor 9

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Abstract

Innate immunity relies on a series of germline-encoded pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), to detect conserved microbial components. TLR9 is typically expressed intracellularly in immune cells such as dendritic cells and recognizes unmethylated bacterial or viral cytosine-phosphate-guanine DNA (CpG-DNA). To investigate innate immune responses through TLR9 signaling pathway in horses, we cloned and characterized equine TLR9. Protein sequence analysis shows that equine TLR9 has a typically conserved cytosolic Toll/Interleukin-1 receptor (TIR) domain, three leucine-rich repeat (LRR) motifs, and more than 82% identity to human, monkey, bovine, canine, feline, porcine and ovine orthologs. Equine TLR9 mRNA was expressed in the spleen, lymph nodes, and peripheral blood leukocytes. Flow cytometric analysis of equine TLR9 expression using a cross-reactive TLR9 mAb identified high constitutive expression of equine TLR9 in PMNs, CD4+ and CD8+ T lymphocytes as well as other leukocytes; similar to human TLR9 expression. The conservation of equine TLR9 and a high expression profile in leukocytes suggests that equine TLR9 is a frequent target for unmethylated CpG-DNA, an essential mechanism for the activation of innate immunity.

Keywords: equine TLR9, PMBC, PMN

1. Introduction

Innate immunity relies on a series of germline-encoded pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), to detect pathogen-associated molecular patterns (PAMPs) (Akira & Takeda, 2004;Akira, Uematsu et al., 2006;Janeway, Jr. & Medzhitov, 2002). TLRs have recently emerged as key PRRs that detect microbial infections and trigger antimicrobial host defense responses. Thus they play a crucial role in the activation of innate immunity and enhance adaptive immunity (Akira & Takeda, 2004;Pasare & Medzhitov, 2004;Takeda, Kaisho et al., 2003;Werling & Jungi, 2003).

Twelve TLRs have been identified in mammals. TLR9 was first cloned from humans and mice (Chuang & Ulevitch, 2000;Hemmi, Takeuchi et al., 2000) and subsequently from domestic animals including swine (Shimosato, Kitazawa et al., 2003), canine (Hashimoto, Asahina et al., 2005), feline (Ignacio, Nordone et al., 2005), bovine and ovine (Griebel, Brownlie et al., 2005). TLR9 belongs to the interleukin-1 receptor/Toll-like receptor superfamily with a conserved cytosolic TIR domain and a few leucine-rich repeat (LRR) motifs (Akira, Uematsu et al., 2006;Bowie & O'Neill, 2000).

TLR9 is mainly expressed in PBMCs and lymphoid tissues such as lymph nodes and the spleen (Chuang & Ulevitch, 2000;Hashimoto, Asahina et al., 2005). In humans, plasmacytoid dendritic cells have the highest level of TLR9 expression (Hornung, Rothenfusser et al., 2002). The TLR9 specific ligand is unmethylated cytosine-phosphate-guanine DNA (CpG-DNA). Evidence to support the CpG-TLR9 interaction includes the finding that TLR9 deficient mice failed to respond to CpG-DNA (Hemmi, Takeuchi et al., 2000). Unmethylated CpG-DNA binds to TLR9, resulting in activation of antigen presenting cells, which subsequently activate T-lymphocytes to secret proinflammatory cytokines such as IFN-γ that promote T-helper type 1 (Th1) immunity. Th1 immunity is essential to control intracellular viral and bacterial infections, and the CpG-TLR9 interaction has been explored as a potential therapy for neoplastic and allergic diseases (Krieg, 2006;Krieg, 2007). Investigations involving TLR9-based immunotherapy for allergic diseases such as asthma have shown that CpG-DNA treatment can inhibit allergic or Th2-driven inflammation (Hayashi & Raz, 2006).

Rhodococcus equi is a facultative intracellular bacterium that can cause fatal bronchopneumonia in foals 2-5 months of age. Clearance of *R. equi* requires IFN-γ from CTL and $CD4^+$ T cells (Hines, Stone et al., 2003;Kohler, Stone et al., 2003;Patton, McGuire et al., 2004;Patton, McGuire et al., 2005). TLR9-based immunotherapy, specifically targeting Th1-induced immunity may provide a therapeutic mechanism to aid in control of *R. equi* infection in young horses. Equine recurrent airway obstruction (RAO) is one of the most common equine respiratory diseases, affecting up to 50% of horses with respiratory diseases worldwide (Bowles, Beadle et al., 2002). RAO is characterized by increased expression of Th2 cytokines such as interleukin (IL)-4 and IL-5, and decreased expression of Th1 cytokines such as IFN-γ in bronchoalveolar immune cells (Bowles, Beadle et al., 2002;Cordeau, Joubert et al., 2004;Dewachi, Joubert et al., 2006;Giguere, Viel et al., 2002;Lavoie, Maghni et al., 2001),

suggesting a shift in the Th1/Th2 balance of affected individuals. TLR9-based immunotherapy may provide a mechanism to restore the Th1/Th2 balance and influence the host allergic response. Prior to application of TLR9-based immunotherapy in horses for either intracellular infections or RAO, it is of fundamental importance to clone and fully characterize equine TLR9. Previous investigations have not included horses as a host for investigation of TLR9 expression with few studies demonstrating potential immunomodulatory effects of CpG-DNA used in the equine host (Flaminio, Borges et al., 2007;Lopez, Hecker et al., 2006;Wattrang, Berg et al., 2005). Here we report the molecular cloning, characterization, and expression of equine TLR9.

2. Materials and Methods

2.1 Primer design for TLR9 cloning

Primers used to clone equine TLR9 were designed based on the homology of TLR9 sequences of human (accession NM_017442), monkey (accession AY788894), bovine (accession AY859726), canine (accession AY859723), feline (accession AY859724), ovine (accession AY859727), swine (accession AY859728), and murine (accession AB045181) hosts.

2.2. Tissue sampling and RNA isolation

All animal protocols were approved by the Kansas State University Institutional Animal Care and Use Committee. Horse tissues were collected from three adult, euthanized quarter horses. After euthanasia, equine tissue samples for RNA isolation were collected immediately, snap frozen in liquid nitrogen and stored at -80 ^{o}C for further total RNA isolation. Samples collected included PBMCs, lymph nodes, spleen, kidney, liver, lung, small intestine, stomach, colon, and heart. Total RNA was isolated from tissues other than PBMCs using TRIzol® reagent (Sigma-Aldrich, St. Louis, MO) or PBMCs using an RNeasy Mini Kit (Qiagen, Valencia, CA), following the protocols with the reagent or kit. Extracted total RNA was also treated with DNase I (Invitrogen, Carlsbad, CA) for 20 to 30 min and purified in RNeasy Mini Kit.

2.3. Rapid amplification of cDNA 5' & 3' ends (RACE)

DNase I-treated total RNA from lymph nodes or PBMCs was used to synthesize cDNA. 3' RACE was performed with FirstChoice® RLM-RACE (Ambion, Austin, TX) and 5' RACE utilized SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA). PCR amplified equine TLR9 fragments were sequenced directly (DNA Sequencing and Genotyping Facility, Kansas State University). Overlapping sequences were analyzed correctly and aligned.

2.4. Cloning and transfection of equine TLR9

The full-length coding sequence of equine TLR9 (GenBank accession no. DQ390541) was amplified by PCR from cDNA with Phusion™ High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) and the following primer pair: CACCATGGGCCCTTGCCATGGTGC (sense) and CTACTCAGCCATCGTCGGGCCCCG (antisense). We then cloned it into a pcDNA™3.1D/V5-His-TOPO® vector (Invitrogen) following the protocol with the pcDNA™3.1 Directional TOPO® Expression Kit. This expression vector has a human cytomegalovirus (CMV) immediate early enhancer/promoter for high-level constitutive expression of the targeted gene and a neomycin resistance gene. Authenticity of equine TRL9 cloning was confirmed by plasmid sequencing. The pcDNA™3.1D/V5-His-TOPO® with equine TLR9 insert was transfected into human embryonic kidney (HEK) 293A cells (Invitrogen) using DOTAP (Roche Applied Science) as the transfection reagent. We chose HEK293 cell line because it has been widely utilized to study the mammalian TLR9 signaling pathway (Bauer, Kirschning et al., 2001;Heil, Hemmi et al., 2004;Heil, hmad-Nejad et al., 2003). The pcDNA™3.1D/V5-His/lacZ, a 8586 bp control vector with the irrelevant gene for β−galactosidase, was utilized as the transfection control. For transfection, 1.5 μg plasmid DNA mixed with 1.5 μl DOTAP was used for 1 x 10^6 293A cells (approximately passage 15) in serum-free media. One hour after transfection (approximately 70% confluence), serum-free media was replaced by complete media (Dulbecco's modified eagle medium with 10% FBS, 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 1% penicillin-streptomycin, Invitrogen), but without geneticin (Invitrogen). After culture for 24 h at 37 ^oC in a humidified sterile incubator with 5% CO_2, geneticin was added (a final concentration

of 400 ug/ml) to induce equine TLR9 expression in 293A cells. Cells were cultured for another 24h before harvesting for total RNA isolation and examination of transient equine TLR9 expression by flow cytometry. We confirmed exclusive equine TLR9 expression in equine TLR9-transfected 293A cells by reverse transcriptase (RT)-PCR using a OneStep RT-PCR kit (Qiagen) and equine TLR9-specific primers (Table 2.1) with 30 cycles of PCR reaction. The RT-PCR or PCR (no RT as control) was performed with 25 ng total RNA in 25 μl reaction plus 0.6 μM final primer concentration for each forward and reverse primer. RT-PCR of 18S and 28S rRNA was performed to serve as internal controls. The cross-species reactivity of a mouse anti-human TLR9 mAb (Imgenex, San diego, CA) with equine TLR9 in equine TLR9-transfected 293A cells was tested by flow cytometry. The endogenous human TLR9 expression in HEK293 cells, with or without transfection, was also detected by flow cytometry as well as RT-PCR using human TLR9-specific primer pair (Table 2.1) with 35 cycles of PCR reaction.

2.5. Quantitative RT-PCR

Primers (Table 2.1) for quantitative RT-PCR were designed using Beacon Designer 4.0 (PREMIER Biosoft International, Palo Alto, CA) for SYBR Green I assay of DNA synthesis. Quantitative PCR was performed on DNase I-treated total RNA samples from individual animals using a OneStep RT-PCR kit (Qiagen) in the presence of 0.2X SYBR Green I (Invitrogen). Quantitative PCR for 18S rRNA (as an internal control) and TLR9 was performed in 25-ul reactions with 50 ng of total RNA and 0.6 μM final primer concentration for each forward and reverse primer, using a Bio-Rad iCycler iQ Real Time PCR System. The real-time PCR specificity was also confirmed by melting curve analysis. The PCR amplification efficiency of TLR9 and 18S rRNA for each reaction was estimated by linear regression (Ramakers, Ruijter et al., 2003), approximately 80% to 100% for TLR9 and 100% for 18S rRNA. The relative gene expression ratio of TLR9 to 18S rRNA was calculated using the formula derived by Schefe et al. (Schefe, Lehmann et al., 2006) and threshold cycle numbers (C_T) that were automatically determined by the Optical System Software (Version 3.1) with the Bio-Rad iCycler iQ Real Time PCR System.

2.6. PMNs and PBMCs isolation

Following skin preparation with 70% ethanol, blood was collected from the jugular vein of healthy young or adult quarter horses into syringes with heparin (50 U/ml). Equine PMNs and PBMCs were isolated by density-gradient separation using Histopaque®-1077 (Sigma-Aldrich). Briefly, blood samples were incubated at room temperature for 30 min to sediment RBCs. The upper phase was transferred gently on the top of Histopaque 1077 in a new tube and centrifuged at 400 g for 30 min. PBMCs, in the cloudy layer, were harvested and transferred to a new tube and washed twice with PBS. The pellet (PMNs) was transferred to a new tube, resuspended in RBC lysis buffer (8.26 g NH_4Cl, 1 g $KHCO_3$ and 0.037 g EDTA in 1 l distilled H_2O, filter-sterilized) for 3 min, and then washed twice with PBS. PMNs were stained with HEMA 3 (Fisher Scientific, Kalamazoo, MI) and examined morphologically. Approximately granulocytes with a yield of 99% were routinely obtained, of which 96% were neutrophils.

2.7. Stimulation of PBMC by PHA

Equine PBMCs were cultured at 1x 10^6/ml in complete media (RPMI 1640 (Hyclone, Logan, UT) with 10% autologous plasma and 1% penicillin-streptomycin) with or without 5 ug/ml PHA (Roche Applied Science, Indianapolis, IN). After culturing for 3 or 4 days at 37 ^oC in a humidified sterile incubator with 5% CO_2, cells were harvested for flow cytometric analysis of TLR9 expression. Cells used for intracellular IFN-γ labeling were treated with Golgiplug™ (protein transport inhibitor Brefeldin A, BD Sciences, San Jose, CA) for 12 hr before harvesting for flow cytometric analysis.

2.8. Flow cytometry

After the mouse anti-human TLR9 mAb (Clone 26C593.2, FITC or PE conjugated, Imgenex) was confirmed to be cross-reactive to equine TLR9 by RT-PCR and flow cytometry, equine TLR9 expression in leukocytes was investigated by flow cytometry, staining with 1 μg mAb/10^6 cells.

Primary mouse anti-equine CD4 (HB61A), anti-equine CD8 (HT14A), anti-swine CD172a (DH59B, for monocyte/granulocyte specific antigen(Tumas, Brassfield et al., 1994)

mAbs were purchased from VMRD (Pullman, WA). Secondary goat anti-mouse Abs, APC-conjugated, were from Invitrogen. PBMCs and PMNs were fixed and permeablized for TLR9 mAb staining after surface labeling for CD4, CD8, and CD172a, following the protocol with the FIX & PERM kit (Invitrogen). The mAbs (Clone MOPC-21, FITC or PE conjugated) used as isotype controls were purchased from BD Sciences.

Intracellular IFN-γ was labeled with mouse anti-bovine IFN-γ-FITC (CC302, AbD Serotec, Raleigh, NC) due to its confirmed cross-species reactivity with equine IFN-γ (Paillot, Daly et al., 2005), following the protocol with the FIX & PERM kit (Invitrogen).

Single-color histograms were generated with CellQuest software (BD Sciences), Two-color dot plot analysis was performed with Winlist 6.0 (Verity Software House, Topsham, ME).

2.8. Statistics

The Kolmogorov-Smirnov (K-S) two sample test (in CellQuest) was used for overlaid histogram comparison. The p-value less than 0.05 is considered significant.

3. Results

3.1. Cloning of equine TLR9

We designed primers based on the homology of mammalian TLR9 DNA sequences and amplified overlapped equine TLR9 fragments from cDNA by PCR. Sequence analysis of these fragments revealed a 3371-bp equine TLR9 gene (GenBank accession no. DQ390541), which included the 5' and 3' un-translated regions as well as the coding sequence of 3096 bp encoding 1031 amino acids. The deduced equine TLR9 protein has more than 82% identity to human, monkey, bovine, canine, feline, swine and ovine orthologs, but less than 75% identity to murine orthologs (Fig. 2.1). It can be inferred that murine TLR9 genes are evolutionarily more distant from the primate TLR9 orthologs when compared with those of domestic mammals. Protein sequence analysis in Conserved Domain Database (Marchler-Bauer, Anderson et al., 2007)

showed equine TLR9 has a typically conserved cytosolic TIR domain and three leucine-rich repeat (LRR) motifs. We blasted the equine TLR9 mRNA sequence in the recently release equine genomic sequence (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9796) that was based on the DNA source of a female thoroughbred horse named "Twilight" from Cornell University in Ithaca, New York. It was determined equine TLR9 is located on the *Equus caballus* (horse) chromosome 16 with one intron between AT and G of the start codon. We surmised that this intron localization is consensus among mammals even though their TLR9 genes are located in different chromosome after we did similar genome blast. A single nucleotide polymorphism (SNP: GAT versus GAC) between quarter horse and thoroughbred breeds was found at base 240 of equine TLR9 coding sequence, even though the TLR9 sequence has not been annotated yet in the released equine genomic sequence.

3.2. Cross-species reactivity of anti-human TLR9 mAb with equine TLR9

The antigenic peptide (268-284 amino acids) of human TLR9 is 82% homologous to equine TLR9. From this finding we reasoned that anti-human TLR9 mAb would likely cross-react with equine TLR9 and the exchange of three out of 17 amino acids in the antigenic peptide would minimally affect its affinity for the anti-human TLR9 mAb. To confirm the cross-species reactivity of anti-human TLR9 mAb, we expressed equine TLR9 protein in HEK293 cells, which have a low level of human TLR9 transcription (Hornung, Rothenfusser et al., 2002;Khvalevsky, Rivkin et al., 2007). We cloned the full-length equine TLR9 coding sequence as described previously and transiently expressed it in 293A cells. Our RT-PCR results (Fig. 2A.) with equine TLR9-specific primers confirmed the exclusive expression of equine TLR9 in 293A cells when transiently transfected with pcDNA™3.1D/V5-His-TOPO® vector plus equine TLR9 insert. We further confirmed this exclusive equine TLR9 expression, a small, but consistent and significant increase of TLR9 expression in 293A cells when transfected with equine TLR9 compared to vector (K-S statistic p-value is less than 0.001 for either the marker areas or the whole histograms), was detected by flow cytometry (Fig. 2.2B). Especially the marked population with TLR9 staining accounted for 53.3% of the equine TLR9-transfected 293A cells, 22.3% higher

than that of the vector-transfected 293A cells. Although the human TLR9 mRNA level was very low (Fig. 2.2C) based on RT-PCR, its protein level in 293A cell was found to be surprisingly high (Fig. 2.2B), which agrees with the reported flow cytometric data using a different anti-human TLR9 Ab (Hoene, Peiser et al., 2006). RT-PCR (Fig. 2.2C) as well as quantitative real time RT-PCR (data not shown) showed no difference in human TLR9 mRNA level among 293A cells, vector-transfected 293A cells and equine TLR9-transfected 293A cells. No difference in human TLR9 expression between 293A cells and vector- transfected 293A cells was found by flow cytometric analysis (data not shown). These data support that anti-human TLR9 mAb cross-reacts with equine TLR9.

3.3. Expression of equine TLR9 in different tissues and immune cell subsets

Quantitative PCR was used to examine TLR9 expression in various tissues. Expression levels were compared among other tissues to that of lymph nodes (arbitrarily set as 100%), 18S ribosomal RNA was used as an internal control. Equine TLR9 is primarily, constitutively expressed in PBMCs, lymph nodes, and the spleen (Fig. 2.3), consistent with the fact that these tissues are enriched with leukocytes. The level of TLR9 in PBMCs is about two to three folds higher than that in lymph nodes and the spleen in which their leukocytes do not account for 100% of its cells in the tissues.

After confirming the cross-species reactivity of human TLR9 mAb with equine TLR9, we examined the specific level of TLR9 expression in specific leukocyte subsets. TLR9 has been shown to be mainly expressed in intracellular compartments (Latz, Schoenemeyer et al., 2004;Leifer, Brooks et al., 2006;Leifer, Kennedy et al., 2004). We reasoned that this also would be the case for equine TLR9. Surface and intracellular labeling of equine TLR9 showed that it was minimally expressed on the plasma membrane and almost exclusively expressed intracellularly in all leukocytes (Fig. 2.4).

We used PHA to enhance TLR9 expression in PBMC. PHA significantly enhanced equine TLR9 expression by PBMC (Fig. 2.5A) because the mitogenic activity of PHA consistently induced pronounced IFN-γ synthesis (Fig. 2.5B) and IFN-γ could subsequently enhance TLR9 expression (Uchijima, Nagata et al., 2005).

We further investigated TLR9 expression in equine leukocyte subsets including PMNs, CD4 and CD8 T cells using two-color flow cytometric analysis. Approximately 90% of the PMNs, CD4 and CD8 T cells exhibited TLR9 expression (Fig. 2.6). This high constitutive expression of TLR9 in leukocytes may suggest an important role for TLR9 in equine innate immunity.

4. Discussion

Since the discovery of the Toll gene for the establishment of dorso-ventral polarity in the developing embryo in *Drosophila* (Hashimoto, Hudson et al., 1988) and its antifungal function in adult flies (Lemaitre, Nicolas et al., 1996), many TLRs, all with highly conserved TIR domains, have been cloned in mammals and shown to play a critical role in linking innate and adaptive immunity (Akira & Takeda, 2004;Janeway, Jr. & Medzhitov, 2002;Pasare & Medzhitov, 2004;Takeda, Kaisho et al., 2003). We report that equine TLR9 is highly homologous to other mammalian orthologs and has a conserved TIR domain and three LRR motifs. Similar to canine and human TLR9 (Chuang & Ulevitch, 2000;Hashimoto, Asahina et al., 2005), equine TLR9 is mainly expressed in peripheral leukocytes and leukocyte enriched tissues such as lymph nodes and the spleen (Fig. 2.3), low level expression exists in other tissues such as heart and lung, which likely reflects minimal leukocyte infiltration.

We confirmed the cross-species reactivity of a mouse anti-human TLR9 mAb to equine TLR9 due to high homology between human and equine TLR9, using both RT-PCR and flow cytometry (Fig. 2.2). In addition, flow cytometry and RT-PCR analyses of HEK293 cells revealed low, constitutive endogenous human TLR9 expression. These data were consistent with previous reports (Hoene, Peiser et al., 2006;Hornung, Rothenfusser et al., 2002;Khvalevsky, Rivkin et al., 2007). The equine TLR9 expression in 293A cells (Fig. 2.2B) after equine TLR9 transfection was consistent.

Although increased TLR9 expression in 293A cells was observed after equine TLR9 transfection, its level was not drastic. Reasons for low equine TLR9 expression in 293A cells following transfection may be explained by a few possibilities. Low, but preexisting endogenous human TLR9 expression in 293A cells accounted for some of the baseline receptor

expression. In addition, the exchange of three out of 17 amino acid residues in the antigenic peptide in equine TLR9 compared to that in human TLR9 may affect affinity to the anti-human TLR9 mAb, resulting in an impaired determination of total TLR9 expression. It is also possible that transfection was not optimal, resulting in a blunted level of equine TLR9 expression. Finally, it should be considered that equine TLR9 expression may have been regulated in 293A cells because TLR9 can bind to CpG motifs in plasmid DNA (Cornelie, Hoebeke et al., 2004). In this instance, plasmid existence is essential for conferring antibiotic resistance in media containing geneticin. If CpG-TLR9 interaction resulted in endocytic plasmid removal, then correspondingly the cell survial in geneticin-containing media could be compromised. This may explain why we could not obtain stable equine TLR9 transfection in 293A cells. However, we were still able to obtain consistent and significant transient equine TLR9 expression in 293A cells as well as in ATCC HEK293 cells (data not shown) to determine the cross-reactivity of an anti-human TLR9 mAb even though HEK293 cells constitutively express low level of human TLR9.

The cross-species reactivity of anti-human TLR9 mAb was exploited to investigate equine TLR9 expression in immune cell subsets using flow cytometry (Fig. 2.4-6). We confirmed that TLR9 expression is almost exclusively expressed and localized intracellularly (Fig. 2.4), presumably limited to the endoplasmic reticulum, endosomes and lysosomes (Latz, Schoenemeyer et al., 2004;Leifer, Brooks et al., 2006;Leifer, Kennedy et al., 2004). Additional studies are needed to confirm equine TLR9 expression in these organelles. Intracellular TLR9 localization reflects the critical need for host clearance of intracellular pathogens (Barton, Kagan et al., 2006) as well as host protection against autoimmune diseases such as systemic lupus erythematosus (Anders, 2005;Rahman & Eisenberg, 2006).

High TLR9 expression was detected within equine PMNs, CD4 and CD8 T cells as well as in other leukocytes (Fig. 2.4 and 2.6). High levels of TLR9 transcription (Hornung, Rothenfusser et al., 2002) have been shown in human plasmacytoid dendritic cells and B cells as well as some level in NK and T cells. Recent data (Fransson, Benson et al., 2007) have demonstrated that human TLR9 protein is expressed in neutrophils, eosinophils, and basophils as well as in monocytes and lymphocytes. Our data are consistent with these findings, although equine immunologic investigations are somewhat limited due to a paucity of equine-specific immunologic reagents.

Constitutive TLR9 expression in both human (Fransson, Benson et al., 2007;Hornung, Rothenfusser et al., 2002) and equine leukocytes presents a similar profile, suggesting the potential similarity in function of TLR9 between humans and horses. Previous investigations have shown that TLR9, especially in professional APCs (B cells, dendritic cells, and macrophages), is involved with host immune response to Herpes simplex virus (Lund, Sato et al., 2003), human papillomavirus (Hasan, Bates et al., 2007), murine cytomegalovirus (Krug, French et al., 2004;Tabeta, Georgel et al., 2004), *Mycobateria tuberculosis* (Bafica, Scanga et al., 2005), *Propionibacterium acnes* (Kalis, Gumenscheimer et al., 2005), and *Trypanosoma cruzi* infection (Bafica, Santiago et al., 2006) through mediating production of proinflammatory cytokines such IL-12 and IFN-γ, and further modulating the host adaptive immune response. Whether equine TLR9 has similar biological function when compared with the human homologue requires further investigation.

Granulocytes, which are primarily composed of neutrophils, constitute a critical component of host innate immunity. Most TLRs, including TLR9, have been found expressed in PMNs (Fransson, Benson et al., 2007;Hayashi, Means et al., 2003;Parker, Whyte et al., 2005;Trevani, Chorny et al., 2003), however, the role of TLR9 expression in this leukocyte subset (as well as T cells) requires further elucidation in most mammalian species including horses. Once TLR9 is fully functionally characterized, the TLR9 agonist may be implemented as a vaccine adjuvant to enhance host Th1 immunity against intracellular pathogens such as *Rhodococcus equi*, or an immunostimulant to attenuate the symptoms of the Th2-biased equine respiratory diseases such as RAO.

In conclusion, our data indicate that equine TLR9 is highly homologous to orthologs in other mammals. It is mainly expressed in the spleen, lymph nodes, and intracellularly in peripheral blood leukocytes. Although equine TLR9 expression is constitutive, it can be enhanced by PHA, likely a result of enhanced IFN-γ secretion by T lymphocytes. The conservation of equine TLR9 and high expression profile in leukocytes may suggest that equine TLR9 is a frequent target by unmethylated CpG-DNA for activation of innate immunity and potentially for enhancement of antigen-specific Th1 immunity against intracellular pathogens.

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References

Akira, S., Takeda, K., 2004. Toll-like receptor signalling. Nat. Rev. Immunol. 4, 499-511.

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783-801.

Anders, H. J., 2005. A Toll for lupus. Lupus 14, 417-422.

Bafica, A., Santiago, H. C., Goldszmid, R., Ropert, C., Gazzinelli, R. T., Sher, A., 2006. Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in Trypanosoma cruzi infection. J. Immunol. 177, 3515-3519.

Bafica, A., Scanga, C. A., Feng, C. G., Leifer, C., Cheever, A., Sher, A., 2005. TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to Mycobacterium tuberculosis. J. Exp. Med. 202, 1715-1724.

Barton, G. M., Kagan, J. C., Medzhitov, R., 2006. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat. Immunol. 7, 49-56.

Bauer, S., Kirschning, C. J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., Lipford, G. B., 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc. Natl. Acad. Sci. U. S. A 98, 9237-9242.

Bowie, A., O'Neill, L. A., 2000. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. J. Leukoc. Biol. 67, 508-514.

Bowles, K. S., Beadle, R. E., Mouch, S., Pourciau, S. S., Littlefield-Chabaud, M. A., Le, B. C., Mistric, L., Fermaglich, D., Horohov, D. W., 2002. A novel model for equine recurrent airway obstruction. Vet. Immunol. Immunopathol. 87, 385-389.

Chuang, T. H., Ulevitch, R. J., 2000. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. Eur. Cytokine Netw. 11, 372-378.

Cordeau, M. E., Joubert, P., Dewachi, O., Hamid, Q., Lavoie, J. P., 2004. IL-4, IL-5 and IFN-gamma mRNA expression in pulmonary lymphocytes in equine heaves. Vet. Immunol. Immunopathol. 97, 87-96.

Cornelie, S., Hoebeke, J., Schacht, A. M., Bertin, B., Vicogne, J., Capron, M., Riveau, G., 2004. Direct evidence that toll-like receptor 9 (TLR9) functionally binds plasmid DNA by specific cytosine-phosphate-guanine motif recognition. J. Biol. Chem. 279, 15124-15129.

Dewachi, O., Joubert, P., Hamid, Q., Lavoie, J. P., 2006. Expression of interleukin (IL)-5 and IL-9 receptors on neutrophils of horses with heaves. Vet. Immunol. Immunopathol. 109, 31-36.

Flaminio, M. J., Borges, A. S., Nydam, D. V., Horohov, D. W., Hecker, R., Matychak, M. B., 2007. The effect of CpG-ODN on antigen presenting cells of the foal. J. Immune. Based. Ther. Vaccines. 5, 1.

Fransson, M., Benson, M., Erjefalt, J. S., Jansson, L., Uddman, R., Bjornsson, S., Cardell, L. O., Adner, M., 2007. Expression of Toll-like receptor 9 in nose, peripheral blood and bone marrow during symptomatic allergic rhinitis. Respir. Res. 8, 17.

Giguere, S., Viel, L., Lee, E., MacKay, R. J., Hernandez, J., Franchini, M., 2002. Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. Vet. Immunol. Immunopathol. 85, 147-158.

Griebel, P. J., Brownlie, R., Manuja, A., Nichani, A., Mookherjee, N., Popowych, Y., Mutwiri, G., Hecker, R., Babiuk, L. A., 2005. Bovine toll-like receptor 9: a comparative analysis of molecular structure, function and expression. Vet. Immunol. Immunopathol. 108, 11-16.

Hasan, U. A., Bates, E., Takeshita, F., Biliato, A., Accardi, R., Bouvard, V., Mansour, M., Vincent, I., Gissmann, L., Iftner, T., Sideri, M., Stubenrauch, F., Tommasino, M., 2007. TLR9 expression and function is abolished by the cervical cancer-associated human papillomavirus type 16. J. Immunol. 178, 3186-3197.

Hashimoto, C., Hudson, K. L., Anderson, K. V., 1988. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. Cell 52, 269-279.

Hashimoto, M., Asahina, Y., Sano, J., Kano, R., Moritomo, T., Hasegawa, A., 2005. Cloning of canine toll-like receptor 9 and its expression in dog tissues. Vet. Immunol. Immunopathol. 106, 159-163.

Hayashi, F., Means, T. K., Luster, A. D., 2003. Toll-like receptors stimulate human neutrophil function. Blood 102, 2660-2669.

Hayashi, T., Raz, E., 2006. TLR9-based immunotherapy for allergic disease. Am. J. Med. 119, 897-6.

Heil, F., Hemmi, H., Hochrein, H., Ampenberger, F., Kirschning, C., Akira, S., Lipford, G., Wagner, H., Bauer, S., 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303, 1526-1529.

Heil, F., hmad-Nejad, P., Hemmi, H., Hochrein, H., Ampenberger, F., Gellert, T., Dietrich, H., Lipford, G., Takeda, K., Akira, S., Wagner, H., Bauer, S., 2003. The Toll-like receptor 7 (TLR7)-specific stimulus loxoribine uncovers a strong relationship within the TLR7, 8 and 9 subfamily. Eur. J. Immunol. 33, 2987-2997.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S., 2000. A Toll-like receptor recognizes bacterial DNA. Nature 408, 740-745.

Hines, S. A., Stone, D. M., Hines, M. T., Alperin, D. C., Knowles, D. P., Norton, L. K., Hamilton, M. J., Davis, W. C., McGuire, T. C., 2003. Clearance of virulent but not avirulent Rhodococcus equi from the lungs of adult horses is associated with intracytoplasmic gamma interferon production by CD4+ and CD8+ T lymphocytes. Clin. Diagn. Lab Immunol. 10, 208-215.

Hoene, V., Peiser, M., Wanner, R., 2006. Human monocyte-derived dendritic cells express TLR9 and react directly to the CpG-A oligonucleotide D19. J. Leukoc. Biol. 80, 1328-1336.

Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, S., Hartmann, G., 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. 168, 4531-4537.

Ignacio, G., Nordone, S., Howard, K. E., Dean, G. A., 2005. Toll-like receptor expression in feline lymphoid tissues. Vet. Immunol. Immunopathol. 106, 229-237.

Janeway, C. A., Jr., Medzhitov, R., 2002. Innate immune recognition. Annu. Rev. Immunol. 20, 197-216.

Kalis, C., Gumenscheimer, M., Freudenberg, N., Tchaptchet, S., Fejer, G., Heit, A., Akira, S., Galanos, C., Freudenberg, M. A., 2005. Requirement for TLR9 in the immunomodulatory activity of Propionibacterium acnes. J. Immunol. 174, 4295-4300.

Khvalevsky, E., Rivkin, L., Rachmilewitz, J., Galun, E., Giladi, H., 2007. TLR3 signaling in a hepatoma cell line is skewed towards apoptosis. J. Cell Biochem. 100, 1301-1312.

Kohler, A. K., Stone, D. M., Hines, M. T., Byrne, B. A., Alperin, D. C., Norton, L. K., Hines, S. A., 2003. Rhodococcus equi secreted antigens are immunogenic and stimulate a type 1 recall response in the lungs of horses immune to R. equi infection. Infect. Immun. 71, 6329-6337.

Krieg, A. M., 2006. Therapeutic potential of Toll-like receptor 9 activation. Nat. Rev. Drug Discov. 5, 471-484.

Krieg, A. M., 2007. Development of TLR9 agonists for cancer therapy. J. Clin. Invest 117, 1184-1194.

Krug, A., French, A. R., Barchet, W., Fischer, J. A., Dzionek, A., Pingel, J. T., Orihuela, M. M., Akira, S., Yokoyama, W. M., Colonna, M., 2004. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. Immunity. 21, 107-119.

Latz, E., Schoenemeyer, A., Visintin, A., Fitzgerald, K. A., Monks, B. G., Knetter, C. F., Lien, E., Nilsen, N. J., Espevik, T., Golenbock, D. T., 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. Nat. Immunol. 5, 190-198.

Lavoie, J. P., Maghni, K., Desnoyers, M., Taha, R., Martin, J. G., Hamid, Q. A., 2001. Neutrophilic airway inflammation in horses with heaves is characterized by a Th2-type cytokine profile. Am. J. Respir. Crit Care Med. 164, 1410-1413.

Leifer, C. A., Brooks, J. C., Hoelzer, K., Lopez, J., Kennedy, M. N., Mazzoni, A., Segal, D. M., 2006. Cytoplasmic targeting motifs control localization of toll-like receptor 9. J. Biol. Chem. 281, 35585-35592.

Leifer, C. A., Kennedy, M. N., Mazzoni, A., Lee, C., Kruhlak, M. J., Segal, D. M., 2004. TLR9 is localized in the endoplasmic reticulum prior to stimulation. J. Immunol. 173, 1179-1183.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M., Hoffmann, J. A., 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell 86, 973-983.

Lopez, A. M., Hecker, R., Mutwiri, G., van Drunen Littel-van den Hurk, Babiuk, L. A., Townsend, H. G., 2006. Formulation with CpG ODN enhances antibody responses to an equine influenza virus vaccine. Vet. Immunol. Immunopathol. 114, 103-110.

Lund, J., Sato, A., Akira, S., Medzhitov, R., Iwasaki, A., 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. J. Exp. Med. 198, 513-520.

Marchler-Bauer, A., Anderson, J. B., Derbyshire, M. K., Weese-Scott, C., Gonzales, N. R., Gwadz, M., Hao, L., He, S., Hurwitz, D. I., Jackson, J. D., Ke, Z., Krylov, D., Lanczycki, C. J., Liebert, C. A., Liu, C., Lu, F., Lu, S., Marchler, G. H., Mullokandov, M., Song, J. S., Thanki, N., Yamashita, R. A., Yin, J. J., Zhang, D., Bryant, S. H., 2007. CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Res. 35, D237-D240.

Page, R. D., 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357-358.

Paillot, R., Daly, J. M., Juillard, V., Minke, J. M., Hannant, D., Kydd, J. H., 2005. Equine interferon gamma synthesis in lymphocytes after in vivo infection and in vitro stimulation with EHV-1. Vaccine 23, 4541-4551.

Parker, L. C., Whyte, M. K., Dower, S. K., Sabroe, I., 2005. The expression and roles of Toll-like receptors in the biology of the human neutrophil. J. Leukoc. Biol. 77, 886-892.

Pasare, C., Medzhitov, R., 2004. Toll-like receptors: linking innate and adaptive immunity. Microbes. Infect. 6, 1382-1387.

Patton, K. M., McGuire, T. C., Fraser, D. G., Hines, S. A., 2004. Rhodococcus equi-infected macrophages are recognized and killed by CD8+ T lymphocytes in a major histocompatibility complex class I-unrestricted fashion. Infect. Immun. 72, 7073-7083.

Patton, K. M., McGuire, T. C., Hines, M. T., Mealey, R. H., Hines, S. A., 2005. Rhodococcus equi-specific cytotoxic T lymphocytes in immune horses and development in asymptomatic foals. Infect. Immun. 73, 2083-2093.

Rahman, A. H., Eisenberg, R. A., 2006. The role of toll-like receptors in systemic lupus erythematosus. Springer Semin. Immunopathol. 28, 131-143.

Ramakers, C., Ruijter, J. M., Deprez, R. H., Moorman, A. F., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62-66.

Schefe, J. H., Lehmann, K. E., Buschmann, I. R., Unger, T., Funke-Kaiser, H., 2006. Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's CT difference" formula. J. Mol. Med. 84, 901-910.

Shimosato, T., Kitazawa, H., Katoh, S., Tomioka, Y., Karima, R., Ueha, S., Kawai, Y., Hishinuma, T., Matsushima, K., Saito, T., 2003. Swine Toll-like receptor 9(1) recognizes CpG motifs of human cell stimulant. Biochim. Biophys. Acta 1627, 56-61.

Tabeta, K., Georgel, P., Janssen, E., Du, X., Hoebe, K., Crozat, K., Mudd, S., Shamel, L., Sovath, S., Goode, J., Alexopoulou, L., Flavell, R. A., Beutler, B., 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. Proc. Natl. Acad. Sci. U. S. A 101, 3516-3521.

Takeda, K., Kaisho, T., Akira, S., 2003. Toll-like receptors. Annu. Rev. Immunol. 21, 335-376.

Trevani, A. S., Chorny, A., Salamone, G., Vermeulen, M., Gamberale, R., Schettini, J., Raiden, S., Geffner, J., 2003. Bacterial DNA activates human neutrophils by a CpG-independent pathway. Eur. J. Immunol. 33, 3164-3174.

Tumas, D. B., Brassfield, A. L., Travenor, A. S., Hines, M. T., Davis, W. C., McGuire, T. C., 1994. Monoclonal antibodies to the equine CD2 T lymphocyte marker, to a pan-granulocyte/monocyte marker and to a unique pan-B lymphocyte marker. Immunobiology 192, 48-64.

Uchijima, M., Nagata, T., Aoshi, T., Koide, Y., 2005. IFN-gamma overcomes low responsiveness of myeloid dendritic cells to CpG DNA. Immunol. Cell Biol. 83, 92-95.

Wattrang, E., Berg, M., Magnusson, M., 2005. Immunostimulatory DNA activates production of type I interferons and interleukin-6 in equine peripheral blood mononuclear cells in vitro. Vet. Immunol. Immunopathol. 107, 265-279.

Werling, D., Jungi, T. W., 2003. TOLL-like receptors linking innate and adaptive immune response. Vet. Immunol. Immunopathol. 91, 1-12.

Table 2.1. Primers for conventional and quantitative real time RT-PCR

Gene	Primer	Sequence (5' → 3')	Amplicon Length (bp)	GenBank accession No.
Equine TLR9	Forward	GTGACTGGCTACCTGGCAAGAC	337	DQ390541
	Reverse	TGGTTATAGAAGTGGCGGTTGTCC		
Human TLR9	Forward	CCACAACAACATCCACAGCCAAGT	266	NM_017442
	Reverse	TCCACCACTTAAAGAAGGCCAGGT		
18S rRNA	Forward	ATGCGGCGGCGTTATTCC	204	AJ311673 NR_003286
	Reverse	GCTATCAATCTGTCAATCCTGTCC		
28S rRNA	Forward	CGGGTAAACGGCGGGAGTAAC	109	NR_003287
	Reverse	TAGGTAGGGACAGTGGGAATCTCG		

FIGURES

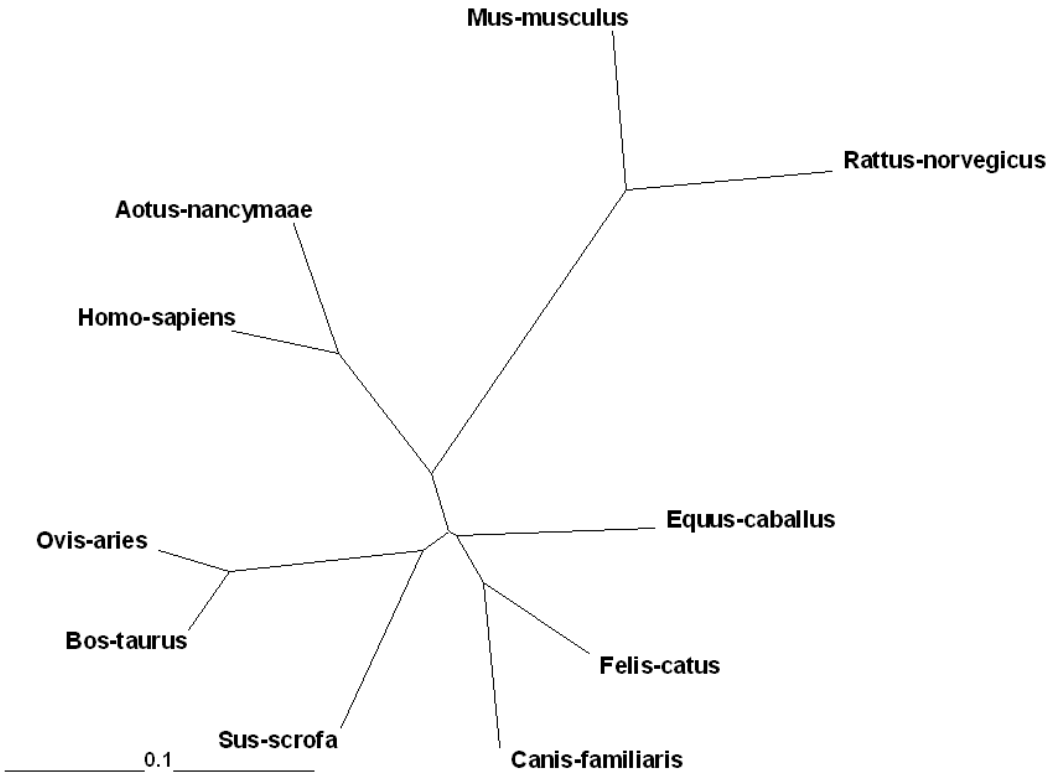
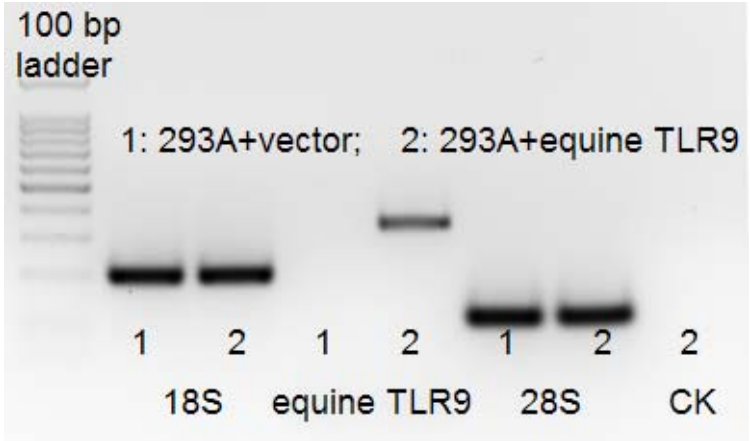


Fig. 2.1. The phylogenetic tree of mammalian TLR9. The unrooted tree was generated using ClustalX program by neighbor-joining method and visualized by Treeview (Page, 1996). Scale bar represents 0.1 amino acid substitution per site.

A



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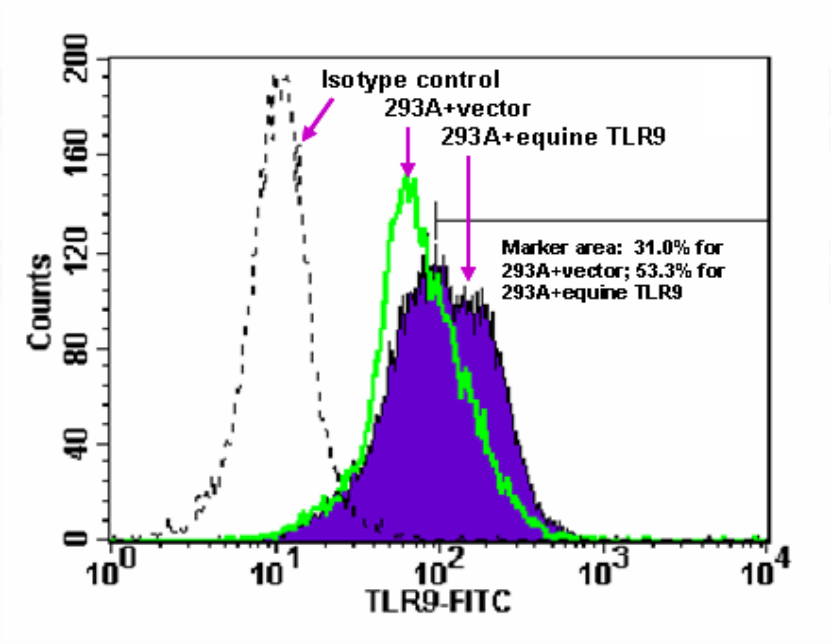


C

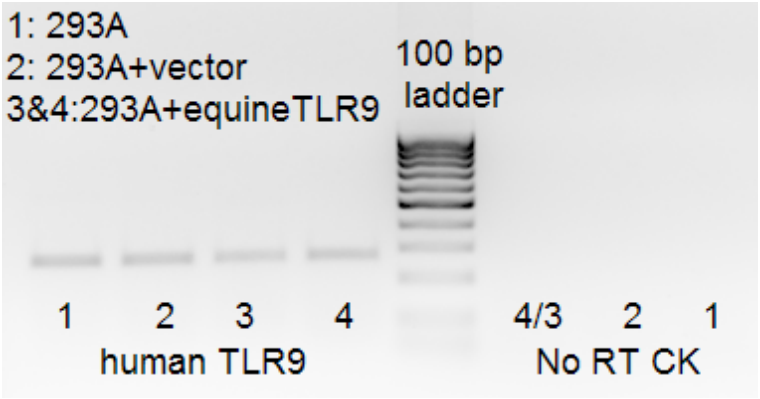


Fig. 2.2. Equine TLR9 was transfected and transiently expressed in 293A cells. "293A+vector" means 293A cells transfected with control vector (The pcDNA™3.1D/V5-His/lacZ), "293A+equine TLR9" means 293A cells transfected with the equine TLR9 insert in the plasmid. In panel A (2% agarose gel picture), 1 is for RNA samples from "293A+vector", 2 is for RNA samples from "293A+equine TLR9". Equine TLR9 expression in 293A cells was confirmed by one-step RT-PCR using a final 0.6 μM gene-specific primers (Table 2.1), 25 ng DNase I-treated total RNA, and 30 PCR cycles in a 25-ul reaction. Total RNA was also confirmed with no DNA contamination by PCR alone (CK: no reverse-transcription control). 18S and 28S ribosomal RNA levels were examined as internal controls. The left lane in panel A is a 100bp ladder (Promega) with 500 bp as the brightest one. In panel B, equine TLR9 was

transfected and transiently expressed in 293A cells and human TLR9 antibody (Imgenex) was confirmed cross-reactive to equine TLR9 by flow cytometry. The marked areas or channels showed the difference in TLR9 staining in 293A cells transfected with the vector or plus equine TLR9. The K-S statistics was used to compare these two overlaid histograms. The p-value is less than 0.001. Similar results were repeated 6 times. In panel C, human TLR9 expression was confirmed by one-step RT-PCR with gene-specific primers and 35 PCR cycles, in which "No RT CK" is for controls with PCR but without reverse-transcription, 3 & 4 are two transfection repeats with plasmids containing the same equine TLR9 insert but from 2 different bacterial colonies. Data for 18S rRNA as equal loading of total RNA are not shown here.

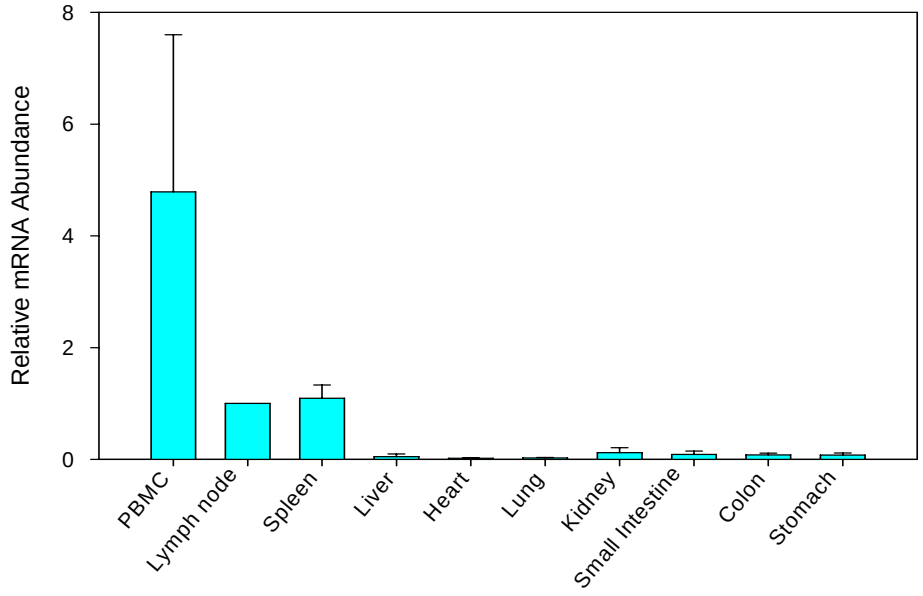


Fig. 2.3. Equine TLR9 is mainly expressed in PBMCs, lymph nodes, and the spleen. TLR9 transcription level in tissues was examined by quantitative one-step RT-PCR with 18S ribosomal RNA as an internal control. TLR9 transcription level was normalized by that in lymph nodes. The relative TLR9 mRNA abundance from three horses was averaged. Error bar shows the standard deviation.

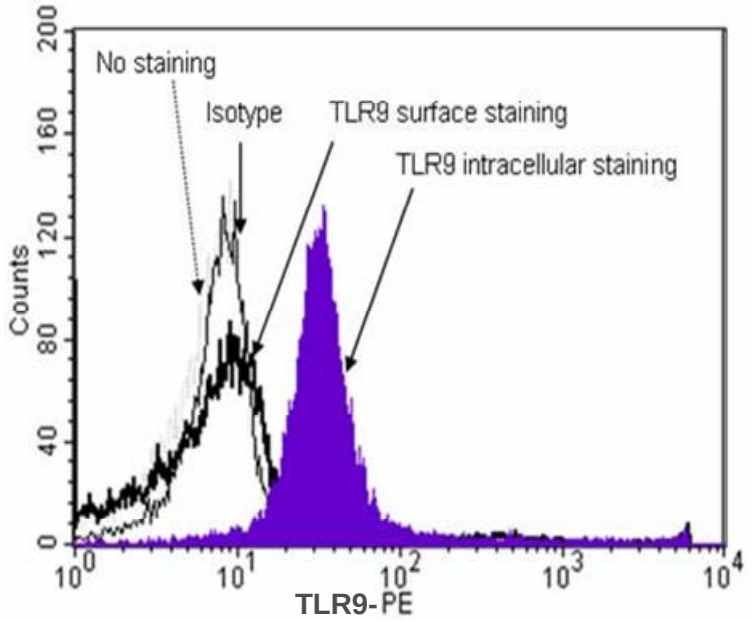
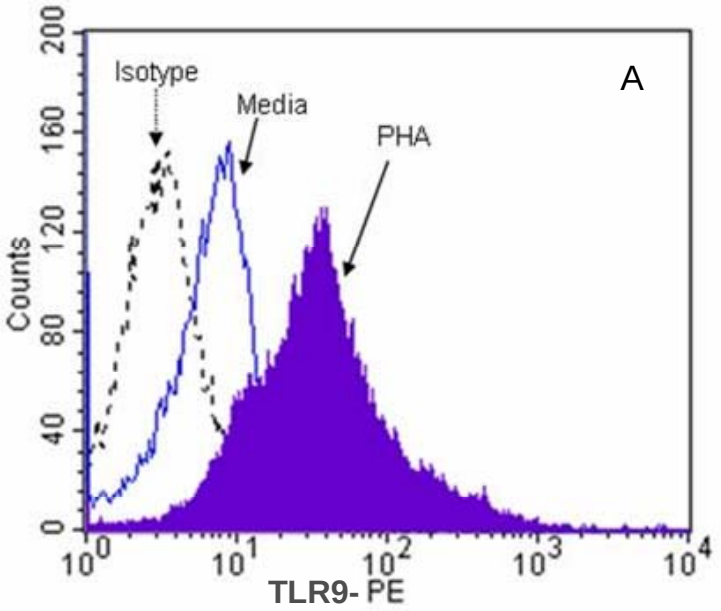


Fig. 2.4. TLR9 is almost exclusively expressed intracellularly.

Heparinized horse blood was incubated at room temperature for 30 min to sediment RBCs. Leukocytes from the plasma were then subject to "TLR9 surface staining" with cross-reactive anti-human TLR9 mAb (PE-conjugated) or fixed and permeablized for "TLR9 intracellular staining" with anti-human TLR9 mAb. The isotype control for TLR9 staining was also PE-conjugated. Similar results were obtained from 6 horses.



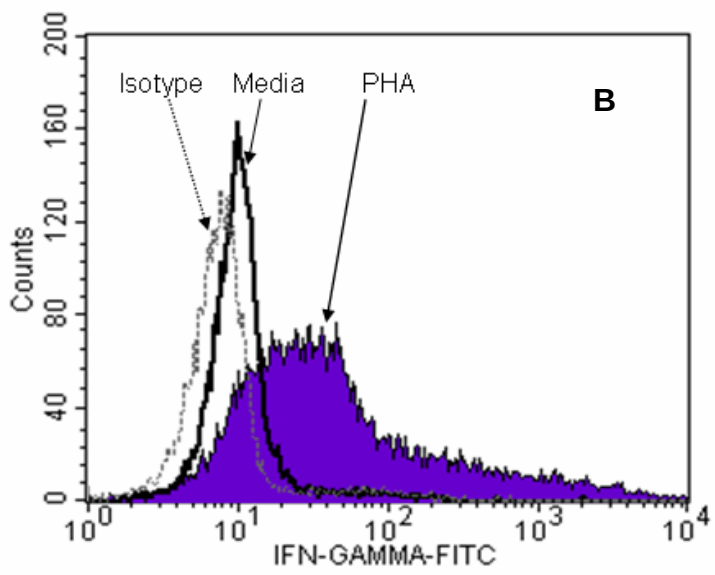
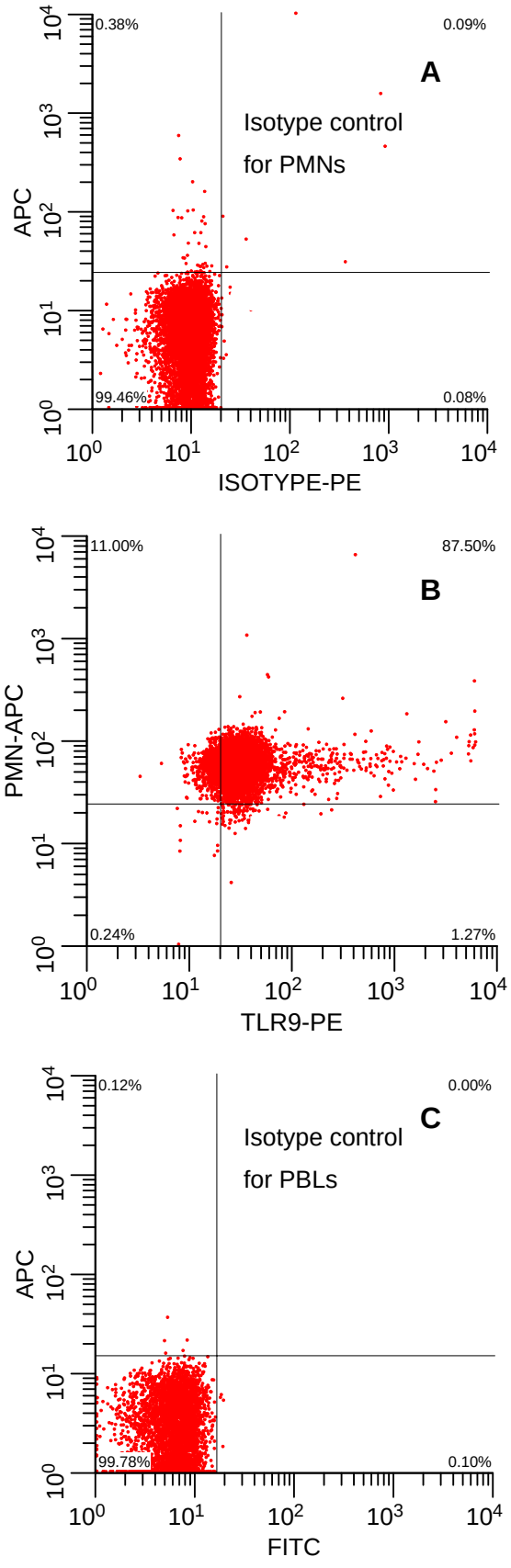


Fig. 2.5. PHA enhanced the expression of equine TLR9 (panel A) and IFN-γ (panel B) determined by flow cytometry. PBMCs were cultured in complete media with and without PHA (5 ug/ ml with 1x 10^6 PBMC /ml complete media) for 4 days before harvest for intracellular TLR9 staining with the PE-conjugated anti-human TLR9 mAb. PBMCs used for IFN-γ staining were treated with Golgiplug for 12 hr and harvested for flow cytometric analysis. Viable cell population was identified by propidium iodide (PI) staining of different PBMCs treated alike because horse viable PBMCs after culture differ from dead ones not only in no PI staining, but also in cell size and granularity. This feature of cultured horse PBMCs remains relatively constant so that the dead cells can be easily gated out. (data not shown). The isotype control for TLR9 staining was PE-conjugated. The isotype control for IFN-γ staining was FITC-conjugated. Similar results were obtained from 6 horses.


A
Isotype control
for PMNs
0.38%
0.09%
99.46%
0.08%
APC
ISOTYPE-PE
B
11.00%
87.50%
0.24%
1.27%
PMN-APC
TLR9-PE
C
Isotype control
for PBLs
0.12%
0.00%
99.78%
0.10%
APC
FITC

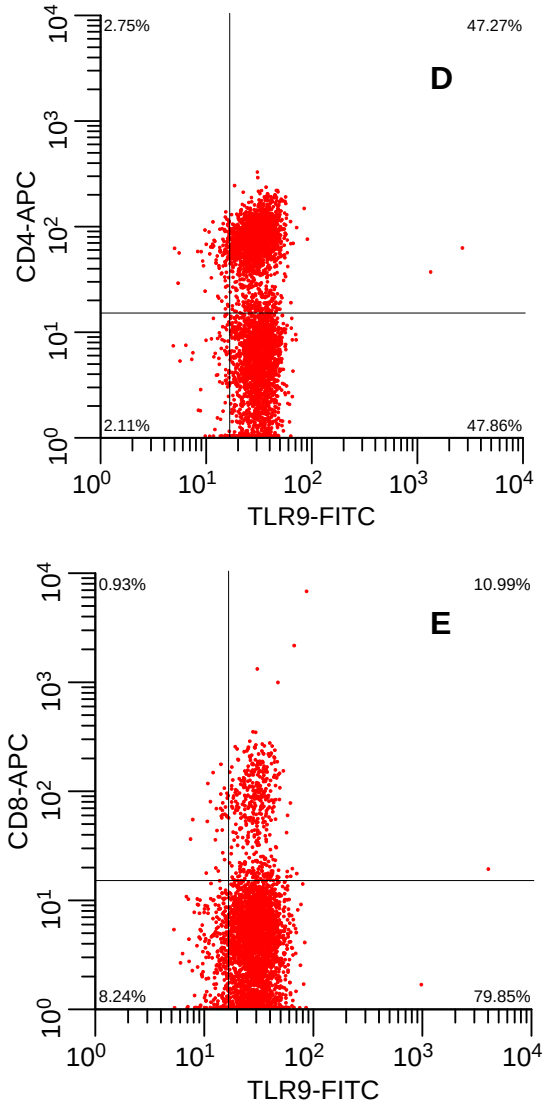


Fig. 2.6. TLR9 is expressed in most equine leukocytes. Leukocytes were surface-stained for PMNs (panel B), CD4 (panel D) and CD8 T cells (panel E), then were fixed and permeablized for TLR9 staining. The anti-human TLR9 mAb was either FITC- or PE-conjugated. Panel A: isotype control for PMNs' TLR9 staining was PE-conjugated; Panel C: isotype control for PBLs' TLR9 staining was FITC-conjugated. In both panel A and C, the APC-congujated secondary Abs were utilized as isotype controls for PMN, CD4, and CD8 surface staining. Percentage of cells in each quadrant is shown for each staining. Similar results were obtained from 8 horses.

CHAPTER 3 - CpG-DNA enhances equine innate immunity

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Abstract

Unmethylated CpG-DNA, the ligand of Toll-like receptor 9, has been extensively tested in mice, humans and many domestic animals for immunological impact and potential as a vaccine adjuvant. Research on CpG-DNA in equine innate immunity remains limited. The impact of CpG-DNA on equine neutrophils has not been reported. To study innate immune responses to CpG-DNA in horses, PMNs, PBMCs and monocyte-derived dendritic cells (moDCs) were stimulated with CpG-DNA and cytokine mRNA expression profiles were investigated using quantitative reverse transcriptase (RT)-PCR. CpG-DNA significantly stimulated IL-8 and TNF-α expression in PMNs, induced expression of IFN-α, IFN-β, IFN-γ, IL-6, IL-8 and IL-12p35 in PBMCs as well as IFN-α and IFN-γ in moDCs. The enhanced expression of IFNs in immune cells by CpG-DNA not only is crucial for host viral clearance, but also may play an important role in mediating host immune responses due to IFNs' anti-inflammatory activity. Tested CpG-DNA showed potential as vaccine adjuvants to enhance CTLs and T helper 1 (Th1) cell-mediated immunity (CMI) against intracellular pathogens because of their apparent induction of type I IFNs and Th1-specific cytokines such as IL-12p35 and IFN-γ. These data provide a basis for further investigation of CpG- DNA as a potentially effective vaccine adjuvant in horses.

Key words: Equine, innate immunity, CpG-DNA, IFN

1. Introduction

Toll-like receptor 9 (TLR9), a highly conserved endosomal pattern recognition receptor (PRR) in mammalian innate immunity, senses unmethylated bacterial or viral cytosine-phosphate-guanine DNA (CpG-DNA) (Kawai and Akira, 2006; Eisenacher et al., 2008; Zhang et al., 2008). Activation of innate immunity through TLR9 signaling can promote the production of interferons (IFN-α, IFN-β & IFN-γ) and proinflammatory cytokines by immune cells from humans, mice and some domestic animals (Hemmi et al., 2000; Hornung et al., 2002; Krieg, 2002; Mutwiri et al., 2003; Wattrang et al., 2005; Hoene et al., 2006; Booth et al., 2007; Dar et al., 2008). These proinflammatory cytokines include IL-1, IL-6, IL-8, IL-12, IL-18 and TNF-α.

Although no agreement on optimal activation of innate immunity by CpG-DNA through TLR9 has been reached, synthetic immunostimulatory CpG-oligodeoxynucleotides (ODNs) have been widely tested for their activation of innate immunity and adjuvant activity for immunization when applied as vaccine adjuvants (Krieg, 2006).

CpG-DNA has showed superior adjuvant activity with many vaccine antigens. CpG-DNA as an adjuvant promotes antigen specific CTLs and Th1 CMI as well as Th1-specific humoral response in murine (Tighe et al., 2000; Welters et al., 2007; Malherbe et al., 2008). Such immune responses are crucial for mammalian hosts to effectively clear viral and other intracellular pathogens.

The biggest challenge for CpG-DNA to be applied as an effective vaccine adjuvant in domestic animals is its species specificity. To overcome such an obstacle, all species of CpG-DNA have to be tested in domestic animals to determine their potential as effective vaccine adjuvants (Rankin et al., 2001; Mutwiri et al., 2003). However, there is no universally accepted system for such a test *in vivo* or *in vitro*. One question that remains to be answered is what are the biomarkers for the activation of innate immunity by CpG-DNA. Type I IFNs and IFN-inducible protein-10 (IP-10/CXCL10) can be considered as the putative biomarkers for CpG-DNA activation of innate immunity (Krieg, 2002; Klinman et al., 2004; Vicari et al., 2007). IFNs have demonstrated remarkable antiviral and antitumor properties as well as having a role in bridging innate and adaptive immunity (Blackwell and Krieg, 2003; Pashenkov et al., 2006; Averett et al., 2007; Thomas et al., 2007; Cooper et al., 2008; Stewart et al., 2008). One potential concern with pronounced induction of immune responses comes from the inflammatory properties of TNF-α and other cytokines. TNF-α stimulation in immune cells may be associated with septic shock (Tracey and Cerami, 1994; Heikenwalder et al., 2004; von Beust et al., 2005; Wang et al., 2007).

As in other mammals, equine TLR9 is mainly expressed intracellularly in all immune cells, including PMNs, T and B cells, monocytes and DCs (Hornung et al., 2002; Flaminio et al., 2007; Zhang et al., 2008). In innate immunity, PMNs play an essential role in the first line of defense against pathogenic infection and tissue damage (Nathan, 2006). PMNs express PRRs to sense microbial pathogen-associated molecular patterns (PAMPs). Appropriate PRR-PAMP interaction, either through direct contact with or via phagocytosis of pathogen and infected host cells may culminate in PMN activation. Recruitment of PMNs to infected or damaged tissue can

initiate a cascade of PMN activation, which further results in recruitment/migration and activation of immature DCs and other immune cells. Thus, PMNs play an essential role in the orchestration of host immune responses (van Gisbergen et al., 2005; Nathan, 2006). However, CpG-DNA stimulation of equine PMNs has not been reported in domestic animals, including horses.

CpG-ODNs 2216 and D19 demonstrated some antiviral activity in an indirect bioassay of IFNs that were induced in equine PBMCs (Wattrang et al., 2005). *In vitro* stimulation by CpG-ODN 2216 on mRNA expression of type I IFNs and IL-12 in PBMCs from two horses was also reported (Wattrang et al., 2005). Potential but non-significant stimulation by CpG-ODN 2135 on mRNA expression of IFN-α and IL-12 in equine moDCs using quantitative RT-PCR was demonstrated (Flaminio et al., 2007). CpG ODN 2007 was tested as an adjuvant in equine influenza virus vaccine and showed improved humoral immune response when compared with conventional vaccine alone (Lopez et al., 2006). Overall, there is a paucity of published data demonstrating CpG-DNA can significantly stimulate equine innate immune response. Whether CpG-DNA can stimulate secretion of IFNs, IL-1β, IL-4, IL-6, IL-8, IL-12, IL-18 and TNF-α from PBMCs, DCs and/or PMNs remains to be demonstrated and is paramount to know prior to *in vivo* testing CpG-DNA as a vaccine adjuvant.

To study equine innate immune responses to CpG-DNA in horses, though arguably through TLR9 signaling pathway, equine PMNs, PBMCs and moDCs were stimulated respectively with CpG-ODNs 2006, 2395 and two other CpG-ODNs we designed. Characterization of immune activation was determined by cytokine expression using quantitative one-step RT-PCR. Our data showed that CpG-DNA significantly stimulated IL-8 and TNF-α production in equine PMNs, enhanced expression of IFN-α, IFN-β, IFN-γ, IL-6, IL-8 and IL-12p35 in PBMCs as well as IFN-α and IFN-γ in moDCs. These tested CpG-DNA species showed antiviral and antitumor properties due to their activation of IFN expression in immune cells. They also showed potential as vaccine adjuvants to enhance CTLs and Th1 CMI against intracellular pathogens because of their apparent induction of type I IFNs and Th1-specific cytokines such as IL-12p35 and IFN-γ.

2. Materials and Methods

2.1. Animals and blood sampling

All animal protocols were approved by the Institutional Animal Care and Use Committee at Kansas State University. Peripheral blood samples (50 ml or less) were collected by jugular venipuncture using heparinzed syringes from randomly-selected healthy Quarter horses and Quarter horse type breeds, 12-25 years of age.

2.2. PBMCs, PMNs isolation and moDCs differentiation

Equine PMNs and PBMCs were isolated by density-gradient separation using Histopaque®-1083 (Sigma-Aldrich, St. Louis, MO) as described (Zhang et al., 2008). To obtain equine moDCs, monocytes were purified from PBMCs by plastic adherence, then were cultured for 5-6 days with recombinant human granulocyte/macrophage colony stimulating factor (GM-CSF) and recombinant equine IL-4 (R&D Systems, Minneapolis, MN), each at 10 ng/ml/10^6 cells (Hammond et al., 1999). Culture media was partly replaced with fresh media with GM-CSF and IL-4 every 2-3 days.

2.3. CpG-ODN design and synthesis

Bacterial genomic DNA has many unmethylated CpG motifs and can stimulate innate immune responses though the TLR9 pathway (Hemmi et al., 2000; Bauer et al., 2001; Dalpke et al., 2006). We also analyzed genomic DNA CpG frequency in these bacteria describe by Dalplke et al. and a *Rhodococcus equi* virulence plasmid (GenBank accession no. AF116907 or AM947677). *Rhodococcus equi* is a facultative intracellular bacterium causing fatal bronchopneumonia in foals. Compared to other bacteria like *Mycobacterium tuberculosis and E. coli*, this *Rhodococcus equi* virulence plasmid has the highest frequency of CpG and could have potential as a vaccine adjuvant through the TLR9 signaling pathway. We extracted two DNA

sequences, designated as RP1 and RP2, with most CpG motifs from this plasmid. The RP1's sequence is TCCCGTCGTTACGCGCTTCCTCGCGTT and RP2's sequence is CTCGCGTCGTTCATCCTGTCGTTCGTG. CpG-ODNs RP1 and RP2 as well as 2006 (TCGTCGTTTTGTCGTTTTGTCGTT) and 2395 (TCGTCGTTTTCGGCGCGCGCCG) (Vollmer et al., 2004) were synthesized and purified by Integrated DNA Technologies (Coralville, IA). All the bases except the last one of these CpG-ODNs were phosphorothioated. CpG-ODN purity was confirmed by mass spectrometry data.

2.4. Cell culture and CpG-DNA stimulation

Complete media for cell culture consisted of RPMI 1640, 10% FBS and 1% penicillin-streptomycin (Hyclone, Logan, UT). Freshly isolated equine PMNs (4 x 10^6/ml) were cultured with or without designed CpG-ODNs (2 μM) for 4 hr before harvest for total RNA isolation. Freshly isolated PBMCs (4 x 10^6/ml) were cultured with or without designated CpG-ODNs (2 μM) for 10 and 24 hr before harvest for total RNA isolation. Following differentiation for 5 to 6 days in complete media with recombinant human GM-CSF and recombinant equine IL-4, moDCs (1 x 10^6/ml) were treated in the presence or absence of CpG-ODNs (2 μM) for 4 and 15 hr before harvest for total RNA isolation. The stimulation time and CpG-ODN concentration for PMNs (Jozsef et al., 2006) and PBMCs (Marshall et al., 2003) were optimized for activation of innate immunity, so was the CpG-ODN concentration. Inactivated/killed West Nile virus (KWNV) was purified from West Nile Innovator® vaccine (Fort Dodge Animal Health Inc., Fort Dodge, IA) by ultracentrifugation (20,000g for 1 hr at 4 ^oC). KWNV (120 ug/ml) was utilized to stimulate PMNs and PBMCs and to determine the effect of KWNV on equine innate immunity *in vitro*. This treatment also was used to compare antiviral activity between KWNV and CpG-DNA.

2.5. Quantitative RT-PCR

Total RNA was isolated from immune cells using an RNeasy Mini Kit (Qiagen, Valencia, CA). Quantitative one-step RT-PCR was performed on DNase I-digested total RNA samples using the QuantiFast SYBR Green RT-PCR Kit (Qiagen Inc, Valencia, CA) in a Bio-Rad iCycler iQ Real Time PCR System (Bio-Rad, Hercules, CA).

Quantitative RT-PCR for 18S rRNA (as the internal control for our experiments) and cytokine expression was performed in 15-ul reactions with 15 ng total RNA and gene-specific primers (Table 3.1). The specificity of all primer pairs was confirmed by sequencing amplicon if unique product was amplified based on its electrophoresis in 1-2% agarose gel, as well as melting-curve analysis in real time RT-PCR. Negative controls without total RNA were included for each run of real time RT-PCR. The PCR amplification efficiency of each reaction was estimated by linear regression, in which 3 to 6 points covering the Ct value were utilized based on the highest correlation coefficiency (Ramakers et al., 2003). The averaged PCR efficiency for each gene was determined for relative gene expression study (Pfaffl, 2001; Cikos et al., 2007).

2.6. Statistical analyses

All mRNA expression data were normalized to 18S rRNA and relative fold change in gene expression was calculated in comparison with control (media/no stimulation). Paired *t*-test for normally distributed data set or Mann-Whitney Rank Sum Test on ranks in SigmaStat 3.5 was performed for all data analyses. $p < 0.05$ is considered statistically significant. Since most of our data were not normally distributed, box-and-whisker plots in Sigmaplot 10 (Systat Software, Inc., San Jose, CA) were applied to show the data, in which error-like bars at the top and bottom are the 95^{th} and 5^{th} percentiles, respectively; the bottom and top of the box are always the 25^{th} and 75^{th} percentiles, respectively. Some extremely high or low data points were plotted as outliers by the box-and-whisker plot program, however, these data points were consistently from the same horses, relatively independent of CpG-DNA stimulation.

3. Results

3.1. Induction of proinflammatory cytokines by CpG-DNA in PMNs

TLR9 is expressed in human and equine PMNs (Fransson et al., 2007; Zhang et al., 2008), thus bacterial DNA and CpG-ODN can activate PMNs through TLR9-dependent signaling pathway even though TLR9-independent pathways may exist (El Kebir et al., 2008;

Fuxman Bass et al., 2008). In our study, purified equine PMNs were stimulated respectively with CpG-ODN 2006, 2395, RP1, RP2 and KWNV for 4 hr (about half of PMN's half life), minimizing the impact of PMN apoptosis during longer time of challenge. After challenge, the expression of IL-1β, IL-6, IL-8 and TNF-α in PMNs were measured by quantitative RT-PCR. Compared to control (media alone), all the tested CpG-ODNs showed significantly ($p = 0.002$) strong stimulation of IL-8 expression in PMNs by 1 to more than 3,000 fold increase (Fig. 3.1). KWNV failed to induce IL-8 expression in PMNs. The variation in PMN's IL-8 expression mainly came from the different responses to CpG-ODN among horses. CpG-ODNs 2006, 2395 and RP2 also showed significant induction of TNF-α expression in PMNs, but this response was weaker than that of IL-8. None of the challenge conditions induced any significant change in expression IL-1β and IL-6 in PMNs (Fig. 3.1).

3.2. Induction of IFNs and proinflammatory cytokines by CpG-DNA in PBMCs

IFNs play a crucial role in host defense against viral infections and neoplasia. In addition, these proteins play a role in bridging innate and adaptive immunity because IFN-α/β promotes antigen cross-presentation by DCs (Whitmire et al., 2005; Havenar-Daughton et al., 2006; Borden et al., 2007; Sadler and Williams, 2008). Another beneficial feature of IFNs is their anti-inflammatory function (Kovarik et al., 2008). Thus, stimulation of IFNs by CpG-DNA in immune cells is of great importance in the evaluation of adjuvant activities of CpG-DNA (Klinman et al., 2004; Krieg, 2007).

In this study, equine PBMCs were stimulated for 10 and 24 hr with CpG-ODNs 2006, 2395, RP1 and RP2 and KWNV to determine induction of IFNs and proinflammatory cytokines. Compared to control, all the CpG-ODNs stimulated significant ($p = 0.002$) expression of IFN-α, IFN-β and IFN-γ at 10 and/or 24 hr stimulation. CpG-ODN 2006 and 2395 induced relatively more IFN-γ expression than CpG-ODNs RP1 and RP2. KWNV induced significant ($p = 0.002$) IFN-α and IFN-γ at both 10 and 24 hr stimulation, but at a lower magnitude when compared to CpG-ODNs (Fig. 3.2).

Among proinflammatory cytokines, induction of Th1 cytokine IL-12 by CpG-ODN in PBMCs was measured as expression of its unique subunit IL-12p35 instead of IL-12p40 because IL-12p40 is also a subunit of Th17 cytokine IL-23 (Oppmann et al., 2000; Weaver et al., 2007;

Tesmer et al., 2008). Compared to control, IL-12p35 expression was enhanced significantly (p = 0.002) at 10 hr stimulation by all the 4 CpG-ODNs and at 24 hr stimulation by CpG-ODNs 2006 and 2395 (Fig.3). The higher IL-12p35 stimulation by CpG-ODNs 2006 and 2395 was consistent with correspondingly higher IFN-γ induction in Fig. 3.2 when compared with CpG-ODNs RP1 and RP2 because Th1 cytokine IL-12 specifically induces IFN-γ expression in NK and T cells (Trinchieri, 2003; Kovarik et al., 2008).

The expression of proinflammatory cytokine IL-6 was induced significantly in PBMCs at both 10 and 24 hr stimulation by CpG-ODN 2006, at 24 hr stimulation by CpG-ODNs 2395, RP1 and RP2 as well as KWNV when compared to control. KWNV induced relative lower IL-6 expression than these CpG-ODNs (Fig. 3.3). Compared to control, IL-8 expression was induced significantly (p = 0.002) at 10 and 24 hr stimulation by all the CpG-ODNs as well as KWNV, indicating the activation status of immune cells. Interestingly, IL-18 expression was significantly (p = 0.002) suppressed in PBMCs by KWNV and CpG-ODN 2006 at 10 hr stimulation when compared to control, other than this, no change in IL-18 expression in PBMCs was identified. Compared to control, the expression of proinflammatory cytokine TNF-α and Th2 cytokine IL-4 in PBMCs was not affected significantly by either CpG-ODNs or KWNV stimulation (Fig. 3.3).

3.3. Induction of IFN-α/γ in moDCs

DCs are crucial professional antigen-presenting cells (APCs) that bridge innate and adaptive immunity and regulate immunity and tolerance via PRRs such as TLRs (Reis e Sousa, 2006). Here, equine moDCs were first differentiated with human GM-CSF and recombinant IL-4, and subsequently stimulated with CpG-ODNs 2006 and 2395. Induction of IFN-α/γ in moDCs by CpG-ODNs was investigated. It was found that CpG-ODNs 2006 and 2395 stimulated significant IFN-α expression ($p \leq 0.001$) at 4 and 15 hr stimulation as well as significant IFN-γ expression (p = 0.026) at 15 hr stimulation when compared to control cells (Fig. 3.4).

4. Discussion

Bacterial DNA or unmethylated CpG-DNA has been researched for a dozen of years for its immunostimulatory effect on immune cells in mammals, leading to its application as a Th1-biased vaccine adjuvant for immunization. Ongoing trials of CpG-ODNs are being carried on in mice, humans and domestic animals. In domestic animals, few *in vivo* and *in vitro* host immune responses to CpG-DNA were investigated. In porcine lymph nodes and PBMCs, *ex- vivo* CpG-ODNs can induce significant production of Th1 cytokines IFNs and IL-12 (Booth et al., 2007). Such host innate immune response to CpG-DNA appears to be essential prior to its application as an effective vaccine adjuvant.

In vitro activation of neutrophils by CpG-DNA has not been previously reported for domestic animals, including horses. In our study, we found that CpG-ODNs 2006 and 2395 and those from *Rhodococcus equi* virulence plasmid activated equine PMNs based upon the expression of IL-8 and TNF-α in PMNs (Fig. 3.1). That some horses consistently showed high up to 3000-fold change in IL-8 mRNA induction by CpG-ODNs probably is because of the autocrine effect of chemokine IL-8 and TLR9-dependent/independent pathways for PMNs' activation by bacterial CpG-DNA (Alvarez et al., 2006; Jozsef et al., 2006). The impact of CpG-ODNs on induction of equine IL-8 protein in PMNs may also need investigation.

PMN activation provided the basis for the migration and activation of immature DCs and other immune cells. Whether all bacterial DNA or other CpG-ODNs activates PMNs from horses or other domestic animals the similar way, through TLR9 signaling pathway, remains to be determined. The lack of significant induction of IL-1β and IL-6 by CpG-ODNs in PMNs was due to low response to CpG-DNA stimulation in two horses at the sampling time. Variability among individuals may prevent us from observing their synchronous peak innate immune response to CpG-DNA challenge, using either PMNs, or PBMCs, or moDC, or other immune cell subsets. Some data points appeared to be outliers in the box-and-whisker plots (Fig. 3.1 to 3.4), but they were consistently from the same horses under any CpG-ODN stimulation. The box-and-whisker plots demonstrated great variation among individuals.

Regardless of the variation in equine innate immune responses to CpG-DNA among individuals, significant induction of type I IFNs, IFN-γ and IL-12 by CpG-ODNs (2006, 2395, RP1 and RP2) was observed in equine PBMCs in our investigation. In addition, IL-6 and IL-8, but not IL-18 and IL-4 demonstrated an increased expression (Fig. 3.2 & 3.3). The reason why KWNV and CpG-ODN 2395 suppressed IL-18 expression in PBMCs after 10 hr stimulation is

unknown. However, this is distinct from the IL-18 stimulation in human PBMCs by the Th2-CMI-promoting Alum adjuvant plus lipopolysaccharide (LPS) (Li et al., 2007), which yet needs proving in horses.

Plasmacytoid DCs are particularly important in host defense against viral infection due to their capability of sensing viral nuclei acids and subsequent IFN production (Gilliet et al., 2008). Due to lack of immunological reagents for purification or differentiation of equine plasmacytoid DCs, moDCs using plastic adherence have been a good alternative for studying equine DC immune function (Hammond et al., 1999), even though the purity of moDCs and control of maturation is problematic. In our study, significant induction of IFN-α and IFN-γ by CpG-ODNs 2006 and 2395 was observed in equine moDCs (Fig. 3.4), indicating these CpG-ODNs' good adjuvant properties. However, significant induction of IL-12-p35 in moDCs was not observed in our challenge with CpG-ODNs (data not shown). An optimal time to measure peak IL-12p35 expression in moDCs when stimulated with CpG-ODNs may have been missed in our experiment, suggesting further investigation is needed.

In general, the biomarkers for equine innate immunity activation by CpG-DNA appeared to be IL-8, IFN-α, IFN-β, and IFN-γ, there was no reason for us to rule out not monitoring the induction of proinflammatory cytokines such as IL-12, IL-1β, IL-6 and TNF-α. Proinflammatory cytokines may exist in the host like a double-edged sword: low level induction of proinflammatory cytokines is essential for effective host immune response, however, high level expression may be deleterious to the host (Tracey and Cerami, 1994; Mann, 2003; Moller and Villiger, 2006; Taqueti et al., 2006; Fogal and Hewett, 2008). We did not observe Th2 cytokine IL-4 stimulation by CpG-ODN in our experiment. In addition, Th17 immune response is found to be highly associated with autoimmune diseases and possibly involved in asthma and allergic diseases (Weaver et al., 2007; Tesmer et al., 2008). Whether CpG-DNA stimulates expression of equine Th17 cytokines such as IL-17 and IL-23 in equine immune cells remains to be determined even though such stimulation was not observed in human (Kattah et al., 2008).

When compared to the relative weaker induction of Th1 cytokines in equine immune cells by KWNV and possibly other vaccine antigens that were not tested here, these tested CpG-ODNs can induce stronger activation of immune cells and a more pronounced Th1-biased innate immunity, thus could promote CTLs and Th1-biased adaptive immunity if applied as vaccine adjuvants. Furthermore, the enhanced expression of IFNs in innate immune cells due to CpG-

DNA not only is crucial for host viral clearance, but also may play an importance role in mediating host immune responses due to their anti-inflammatory functions against possibly exuberant proinflammatory cytokines such as IL-1β and TNF-α (Borden et al., 2007; Kovarik et al., 2008). These data provide a legitimate reason for investigation of these CpG-ODNs as potentially effective vaccine adjuvants in horses.

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References

Alvarez, M.E., Fuxman Bass, J.I., Geffner, J.R., Calotti, P.X., Costas, M., Coso, O.A., Gamberale, R., Vermeulen, M.E., Salamone, G., Martinez, D., Tanos, T., Trevani, A.S., 2006. Neutrophil signaling pathways activated by bacterial DNA stimulation. J. Immunol. 177, 4037-4046.

Averett, D.R., Fletcher, S.P., Li, W., Webber, S.E., Appleman, J.R., 2007. The pharmacology of endosomal TLR agonists in viral disease. Biochem. Soc. Trans. 35, 1468-1472.

Bauer, S., Kirschning, C.J., Hacker, H., Redecke, V., Hausmann, S., Akira, S., Wagner, H., Lipford, G.B., 2001. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc. Natl. Acad. Sci. U. S. A. 98, 9237-9242.

Blackwell, S.E., Krieg, A.M., 2003. CpG-A-induced monocyte IFN-gamma-inducible protein-10 production is regulated by plasmacytoid dendritic cell-derived IFN-alpha. J. Immunol. 170, 4061-4068.

Booth, J.S., Nichani, A.K., Benjamin, P., Dar, A., Krieg, A.M., Babiuk, L.A., Mutwiri, G.K., 2007. Innate immune responses induced by classes of CpG oligodeoxynucleotides in ovine lymph node and blood mononuclear cells. Vet. Immunol. Immunopathol. 115, 24-34.

Borden, E.C., Sen, G.C., Uze, G., Silverman, R.H., Ransohoff, R.M., Foster, G.R., Stark, G.R., 2007. Interferons at age 50: past, current and future impact on biomedicine. Nat Rev Drug Discov 6, 975-990.

Cikos, S., Bukovska, A., Koppel, J., 2007. Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. BMC Mol Biol 8, 113.

Cooper, C.L., Ahluwalia, N.K., Efler, S.M., Vollmer, J., Krieg, A.M., Davis, H.L., 2008. Immunostimulatory effects of three classes of CpG oligodeoxynucleotides on PBMC from HCV chronic carriers. J Immune Based Ther Vaccines 6, 3.

Dalpke, A., Frank, J., Peter, M., Heeg, K., 2006. Activation of toll-like receptor 9 by DNA from different bacterial species. Infect. Immun. 74, 940-946.

Dar, A., Nichani, A.K., Benjamin, P., Lai, K., Soita, H., Krieg, A.M., Potter, A., Babiuk, L.A., Mutwiri, G.K., 2008. Attenuated cytokine responses in porcine lymph node cells stimulated with CpG DNA are associated with low frequency of IFNalpha-producing cells and TLR9 mRNA expression. Vet. Immunol. Immunopathol. 123, 324-336.

Eisenacher, K., Steinberg, C., Reindl, W., Krug, A., 2008. The role of viral nucleic acid recognition in dendritic cells for innate and adaptive antiviral immunity. Immunobiology 212, 701-714.

El Kebir, D., Jozsef, L., Filep, J.G., 2008. Neutrophil recognition of bacterial DNA and Toll-like receptor 9-dependent and -independent regulation of neutrophil function. Arch. Immunol. Ther. Exp. (Warsz). 56, 41-53.

Flaminio, M.J., Borges, A.S., Nydam, D.V., Horohov, D.W., Hecker, R., Matychak, M.B., 2007. The effect of CpG-ODN on antigen presenting cells of the foal. J Immune Based Ther Vaccines 5, 1.

Fogal, B., Hewett, S.J., 2008. Interleukin-1beta: a bridge between inflammation and excitotoxicity? J. Neurochem. 106, 1-23.

Fransson, M., Benson, M., Erjefalt, J.S., Jansson, L., Uddman, R., Bjornsson, S., Cardell, L.O., Adner, M., 2007. Expression of Toll-like receptor 9 in nose, peripheral blood and bone marrow during symptomatic allergic rhinitis. Respir Res 8, 17.

Fuxman Bass, J.I., Gabelloni, M.L., Alvarez, M.E., Vermeulen, M.E., Russo, D.M., Zorreguieta, A., Geffner, J.R., Trevani, A.S., 2008. Characterization of bacterial DNA binding to human neutrophil surface. Lab. Invest. 88, 926-937.

Gilliet, M., Cao, W., Liu, Y.J., 2008. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol 8, 594-606.

Hammond, S.A., Horohov, D., Montelaro, R.C., 1999. Functional characterization of equine dendritic cells propagated ex vivo using recombinant human GM-CSF and recombinant equine IL-4. Vet. Immunol. Immunopathol. 71, 197-214.

Havenar-Daughton, C., Kolumam, G.A., Murali-Krishna, K., 2006. Cutting Edge: The direct action of type I IFN on CD4 T cells is critical for sustaining clonal expansion in response to a viral but not a bacterial infection. J. Immunol. 176, 3315-3319.

Heikenwalder, M., Polymenidou, M., Junt, T., Sigurdson, C., Wagner, H., Akira, S., Zinkernagel, R., Aguzzi, A., 2004. Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. Nat. Med. 10, 187-192.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., Akira, S., 2000. A Toll-like receptor recognizes bacterial DNA. Nature 408, 740-745.

Hoene, V., Peiser, M., Wanner, R., 2006. Human monocyte-derived dendritic cells express TLR9 and react directly to the CpG-A oligonucleotide D19. J. Leukoc. Biol. 80, 1328-1336.

Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, S., Hartmann, G., 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. 168, 4531-4537.

Jozsef, L., Khreiss, T., El Kebir, D., Filep, J.G., 2006. Activation of TLR-9 induces IL-8 secretion through peroxynitrite signaling in human neutrophils. J. Immunol. 176, 1195-1202.

Kattah, M.G., Wong, M.T., Yocum, M.D., Utz, P.J., 2008. Cytokines secreted in response to Toll-like receptor ligand stimulation modulate differentiation of human Th17 cells. Arthritis Rheum. 58, 1619-1629.

Kawai, T., Akira, S., 2006. TLR signaling. Cell Death Differ. 13, 816-825.

Klinman, D.M., Currie, D., Gursel, I., Verthelyi, D., 2004. Use of CpG oligodeoxynucleotides as immune adjuvants. Immunol. Rev. 199, 201-216.

Kovarik, P., Sauer, I., Schaljo, B., 2008. Molecular mechanisms of the anti-inflammatory functions of interferons. Immunobiology 212, 895-901.

Krieg, A.M., 2002. CpG motifs in bacterial DNA and their immune effects. Annu. Rev. Immunol. 20, 709-760.

Krieg, A.M., 2006. Therapeutic potential of Toll-like receptor 9 activation. Nat Rev Drug Discov 5, 471-484.

Krieg, A.M., 2007. Development of TLR9 agonists for cancer therapy. J. Clin. Invest. 117, 1184-1194.

Li, H., Nookala, S., Re, F., 2007. Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1beta and IL-18 release. J. Immunol. 178, 5271-5276.

Lopez, A.M., Hecker, R., Mutwiri, G., van Drunen Littel-van den Hurk, S., Babiuk, L.A., Townsend, H.G., 2006. Formulation with CpG ODN enhances antibody responses to an equine influenza virus vaccine. Vet. Immunol. Immunopathol. 114, 103-110.

Malherbe, L., Mark, L., Fazilleau, N., McHeyzer-Williams, L.J., McHeyzer-Williams, M.G., 2008. Vaccine adjuvants alter TCR-based selection thresholds. Immunity 28, 698-709.

Mann, D.L., 2003. Stress-activated cytokines and the heart: from adaptation to maladaptation. Annu. Rev. Physiol. 65, 81-101.

Marshall, J.D., Fearon, K., Abbate, C., Subramanian, S., Yee, P., Gregorio, J., Coffman, R.L., Van Nest, G., 2003. Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. J. Leukoc. Biol. 73, 781-792.

Moller, B., Villiger, P.M., 2006. Inhibition of IL-1, IL-6, and TNF-alpha in immune-mediated inflammatory diseases. Springer Semin. Immunopathol. 27, 391-408.

Mutwiri, G., Pontarollo, R., Babiuk, S., Griebel, P., van Drunen Littel-van den Hurk, S., Mena, A., Tsang, C., Alcon, V., Nichani, A., Ioannou, X., Gomis, S., Townsend, H., Hecker, R., Potter, A., Babiuk, L.A., 2003. Biological activity of immunostimulatory CpG DNA motifs in domestic animals. Vet. Immunol. Immunopathol. 91, 89-103.

Nathan, C., 2006. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol 6, 173-182.

Oppmann, B., Lesley, R., Blom, B., Timans, J.C., Xu, Y., Hunte, B., Vega, F., Yu, N., Wang, J., Singh, K., Zonin, F., Vaisberg, E., Churakova, T., Liu, M., Gorman, D., Wagner, J., Zurawski, S., Liu, Y., Abrams, J.S., Moore, K.W., Rennick, D., de Waal-Malefyt, R.,

Hannum, C., Bazan, J.F., Kastelein, R.A., 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 13, 715-725.

Pashenkov, M., Goess, G., Wagner, C., Hormann, M., Jandl, T., Moser, A., Britten, C.M., Smolle, J., Koller, S., Mauch, C., Tantcheva-Poor, I., Grabbe, S., Loquai, C., Esser, S., Franckson, T., Schneeberger, A., Haarmann, C., Krieg, A.M., Stingl, G., Wagner, S.N., 2006. Phase II trial of a toll-like receptor 9-activating oligonucleotide in patients with metastatic melanoma. J. Clin. Oncol. 24, 5716-5724.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29, e45.

Ramakers, C., Ruijter, J.M., Deprez, R.H., Moorman, A.F., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62-66.

Rankin, R., Pontarollo, R., Ioannou, X., Krieg, A.M., Hecker, R., Babiuk, L.A., van Drunen Littel-van den Hurk, S., 2001. CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. Antisense Nucleic Acid Drug Dev. 11, 333-340.

Reis e Sousa, C., 2006. Dendritic cells in a mature age. Nat Rev Immunol 6, 476-483.

Sadler, A.J., Williams, B.R., 2008. Interferon-inducible antiviral effectors. Nat Rev Immunol 8, 559-568.

Stewart, V.A., McGrath, S., Krieg, A.M., Larson, N.S., Angov, E., Smith, C.L., Brewer, T.G., Heppner, D.G., Jr., 2008. Activation of innate immunity in healthy Macaca mulatta macaques by a single subcutaneous dose of GMP CpG 7909: safety data and interferon-inducible protein-10 kinetics for humans and macaques. Clin Vaccine Immunol 15, 221-226.

Taqueti, V.R., Mitchell, R.N., Lichtman, A.H., 2006. Protecting the pump: controlling myocardial inflammatory responses. Annu. Rev. Physiol. 68, 67-95.

Tesmer, L.A., Lundy, S.K., Sarkar, S., Fox, D.A., 2008. Th17 cells in human disease. Immunol. Rev. 223, 87-113.

Thomas, A., Laxton, C., Rodman, J., Myangar, N., Horscroft, N., Parkinson, T., 2007. Investigating Toll-like receptor agonists for potential to treat hepatitis C virus infection. Antimicrob. Agents Chemother. 51, 2969-2978.

Tighe, H., Takabayashi, K., Schwartz, D., Marsden, R., Beck, L., Corbeil, J., Richman, D.D., Eiden, J.J., Jr., Spiegelberg, H.L., Raz, E., 2000. Conjugation of protein to immunostimulatory DNA results in a rapid, long-lasting and potent induction of cell-mediated and humoral immunity. Eur. J. Immunol. 30, 1939-1947.

Tracey, K.J., Cerami, A., 1994. Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. Annu. Rev. Med. 45, 491-503.

Trinchieri, G., 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 3, 133-146.

van Gisbergen, K.P., Geijtenbeek, T.B., van Kooyk, Y., 2005. Close encounters of neutrophils and DCs. Trends Immunol 26, 626-631.

Vicari, A.P., Schmalbach, T., Lekstrom-Himes, J., Morris, M.L., Al-Adhami, M.J., Laframboise, C., Leese, P., Krieg, A.M., Efler, S.M., Davis, H.L., 2007. Safety, pharmacokinetics and immune effects in normal volunteers of CPG 10101 (ACTILON), an investigational synthetic toll-like receptor 9 agonist. Antivir Ther 12, 741-751.

Vollmer, J., Weeratna, R., Payette, P., Jurk, M., Schetter, C., Laucht, M., Wader, T., Tluk, S., Liu, M., Davis, H.L., Krieg, A.M., 2004. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. Eur. J. Immunol. 34, 251-262.

von Beust, B.R., Johansen, P., Smith, K.A., Bot, A., Storni, T., Kundig, T.M., 2005. Improving the therapeutic index of CpG oligodeoxynucleotides by intralymphatic administration. Eur. J. Immunol. 35, 1869-1876.

Wang, L., Jiang, W., Ding, G., Cao, H., Lu, Y., Luo, P., Zhou, H., Zheng, J., 2007. The newly identified CpG-N ODN208 protects mice from challenge with CpG-S ODN by decreasing TNF-alpha release. Int Immunopharmacol 7, 646-655.

Wattrang, E., Berg, M., Magnusson, M., 2005. Immunostimulatory DNA activates production of type I interferons and interleukin-6 in equine peripheral blood mononuclear cells in vitro. Vet. Immunol. Immunopathol. 107, 265-279.

Weaver, C.T., Hatton, R.D., Mangan, P.R., Harrington, L.E., 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu. Rev. Immunol. 25, 821-852.

Welters, M.J., Bijker, M.S., van den Eeden, S.J., Franken, K.L., Melief, C.J., Offringa, R., van der Burg, S.H., 2007. Multiple CD4 and CD8 T-cell activation parameters predict

vaccine efficacy in vivo mediated by individual DC-activating agonists. Vaccine 25, 1379-1389.

Whitmire, J.K., Benning, N., Whitton, J.L., 2005. Cutting edge: early IFN-gamma signaling directly enhances primary antiviral CD4+ T cell responses. J. Immunol. 175, 5624-5628.

Zhang, Y.W., Davis, E.G., Blecha, F., Wilkerson, M.J., 2008. Molecular cloning and characterization of equine Toll-like receptor 9. Vet. Immunol. Immunopathol. 124, 209-219.

Table 3.1 Primers designed for quantitative RT-PCR.

Gene	Primer	Sequence (5' to 3')	Amplicon Length (bp)	GenBank accession No.
18S rRNA	Forward	ATGCGGCGGCGTTATTCC	204	AJ311673
	Reverse	GCTATCAATCTGTCAATCCTGTCC		
IFN-α	Forward	CGGAAGCCTCAAGCCATCTC	225	M14540, M14542,
	Reverse	GTATCTCCTCACAGCCAGCAG		M14543, M14544
IFN-β	Forward	TGAGCAAGCACAGCAGTTCC	160	A33685
	Reverse	GCCAAGTTCCTGAGCATTTCC		
IFN-γ	Forward	GCCAAATCGTCTCCTTCTACTTC	260	D28520
	Reverse	CTGACTCCTCTTCCGCTTCC		
IL-1β	Forward	AAGGAGAAGAAGAGACTGAC	238	U92481
	Reverse	GCTTGAGAGGTGCTGATG		
IL-4	Forward	GAACAACCTCACAGATGGAAAG	238	AF305617
	Reverse	GCTCTTCTTGGCTTCATTCAC		
IL-6	Forward	AAGATGAAGAATCCAGAAGTAACC	219	U64794
	Reverse	CTGACCAGAGGAAGGAATGC		
IL-8	Forward	GCCTTGGTTTCTTCTTTAATC	253	AY184956
	Reverse	CACTCTAACATCATTACATTATCC		
IL-12p35	Forward	GAGGACCGTCAGCAACAC	233	Y11130
	Reverse	ACAGCGTCATCATAGAAGAGG		
IL-18	Forward	TCAGATTACTTTGGCAGGCTTG	212	Y11131
	Reverse	TCACACTTCACAGAGATGGTTAC		
TNF-α	Forward	TTGTGCCTCAGCCTCTTC	109	AB035735
	Reverse	CATTCGGTAACTGCTCTTCC		

Figures

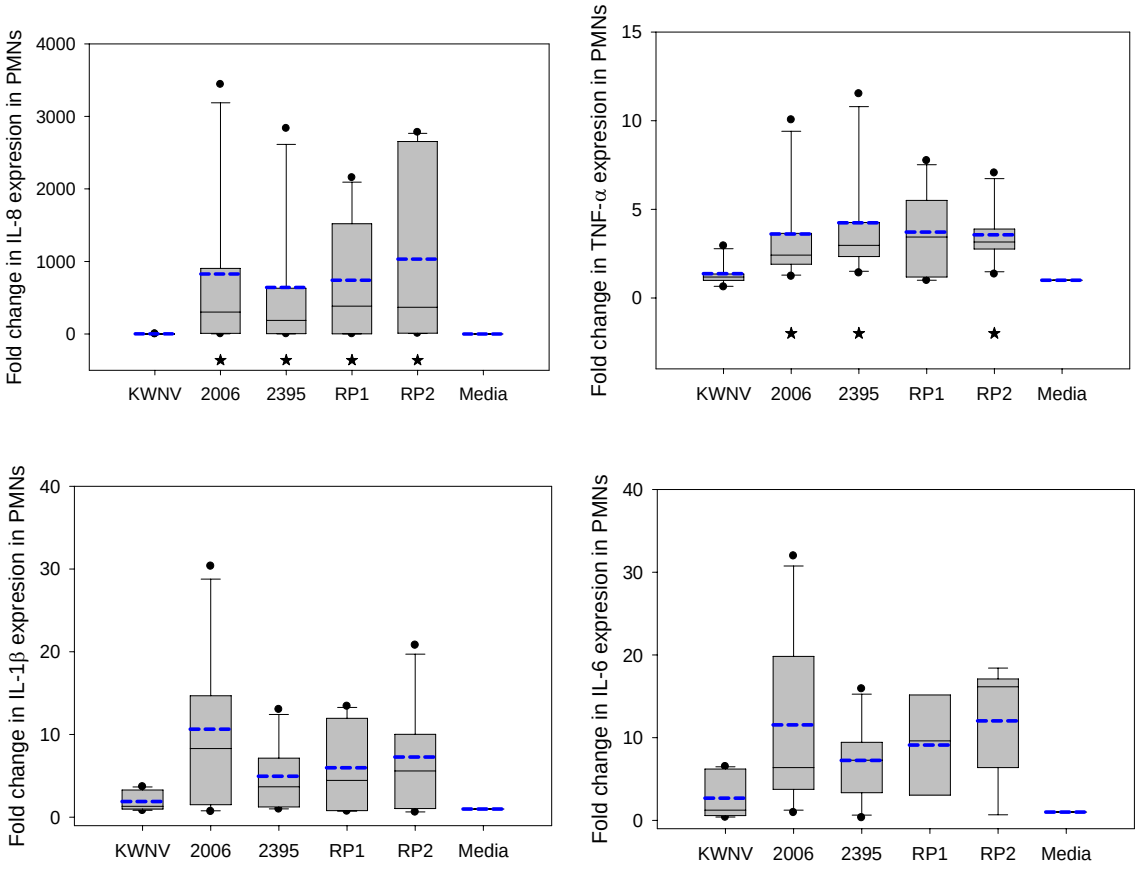


Fig. 3.1. Relative fold changes in expression of IL-8, TNF-α, IL-1β and IL-6 in equine PMNs due to stimulation by KWNV and CpG-ODNs 2006, 2395, RP1 and RP2 when compared to control (media). Freshly isolated PMNs were cultured for 4 hr in complete medium at 4 x 10^6 cells/ml, in the presence or absence of KWNV and CpG-ODNs. The cytokine gene expression was measured by one-step quantitative RT-PCR, using 18S rRNA as the internal control for mRNA data normalization. The relative gene expression as fold change was calculated in comparison with control (n=6). The control's relative gene expression was 1 and of no fold change. The stars indicated a statistically significant difference (p = 0.002) between control and stimulation. The dashed thick blue bar is for the mean, the thin black bar for the median. In the box-and-whisker plot, some data points appeared to be outliers (black dots), but they were consistently shown from the same horses under any CpG-ODN stimulation. The box-and-whisker plot, regardless of statistical distribution, just showed great variation among individuals (same for the rest of figures).

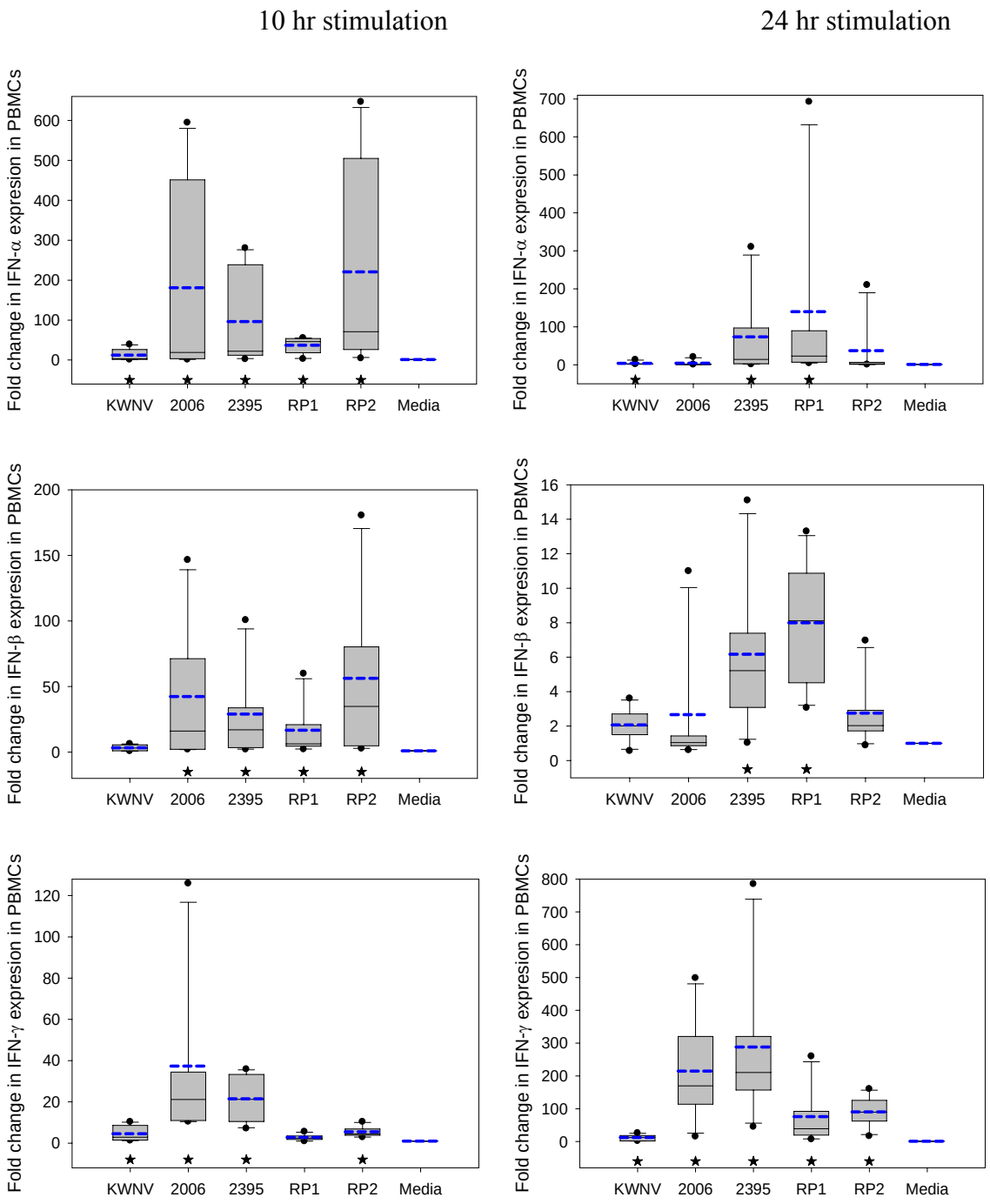
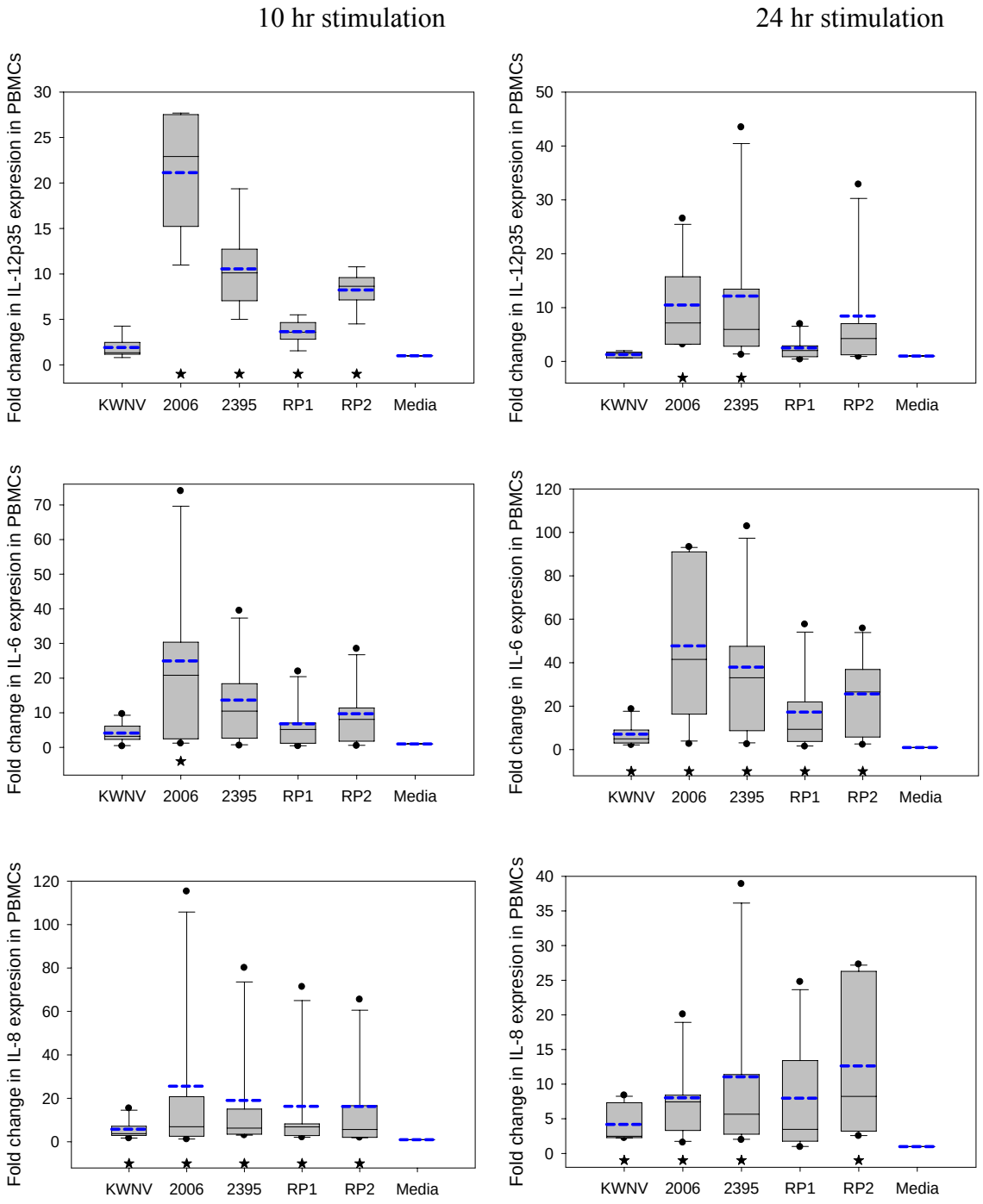


Fig. 3.2. Relative fold changes in expression of IFN-α, IFN-β and IFN-γ in equine PBMCs due to stimulation by KWNV and CpG-ODNs 2006, 2395, RP1 and RP2 when compared to control (n=6). Freshly isolated PBMCs were cultured for 10 hr and 24 hr in complete medium at 4 x 10^6 cells/ml, in the presence or absence of KWNV and CpG-ODNs. The mRNA expression of IFNs was analyzed as in Fig. 3.1. The control's relative gene expression was 1 and of no fold

change. The stars indicated a statistically significant difference ($p = 0.002$) between control and stimulation. The dashed thick blue bar is the mean, the thin black bar is the median.



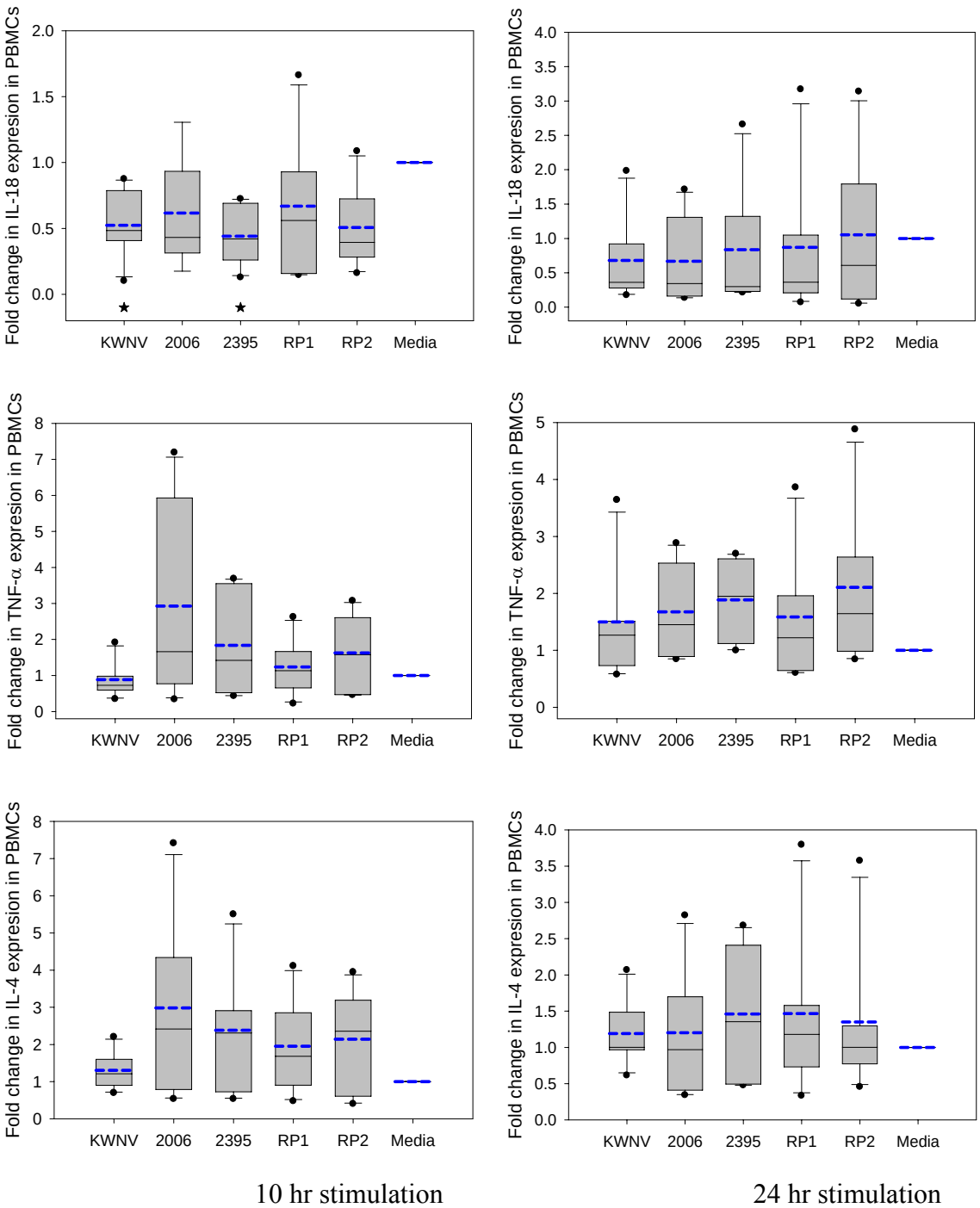
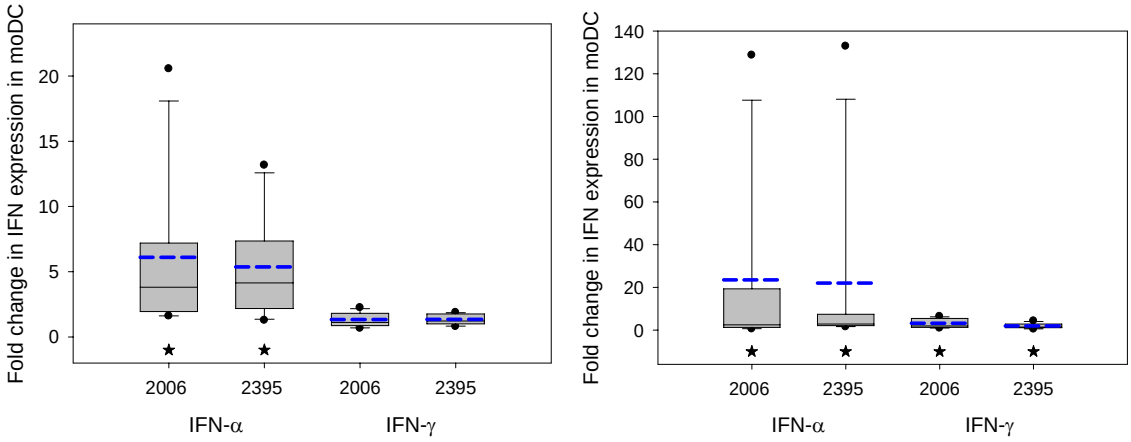


Fig. 3.3. Relative fold changes in expression of proinflammatory cytokines IL12p35, IL-6, IL-8, IL-18, TNF-α and IL-4 in equine PBMCs due to stimulation by KWNV and CpG-ODNs 2006, 2395, RP1 and RP2 when compared to control (n=6). Total RNA was from the same samples as in Fig. 3.2. The mRNA expression of proinflammatory cytokines was analyzed

as in Fig. 3.1. The control's relative gene expression was 1 and of no fold change. The stars indicated a statistically significant difference ($p = 0.002$) between control and stimulation. The dashed thick blue bar is the mean, the thin black bar is the median.



4 hr stimulation 15 hr stimulation

Fig. 3.4. Relative fold changes in expression of IFN-α and IFN-γ in equine moDCs due to stimulation by CpG-ODNs 2006 and 2395 when compared to control (n=7). Equine moDCs were differentiated with recombinant human GM-CSF and recombinant equine IL-4 from monocytes. The differentiated equine moDCs were cultured for 4 hr and 15 hr in complete medium at 1 x 10^6 cells/ml, in the presence or absence of CpG-ODNs. The mRNA expression of IFNs was analyzed as in Fig. 3.1. The control's relative gene expression was 1 and of no fold change (not shown in the box and whisker plots). The stars indicated a statistically significant difference ($p \leq 0.001$ for IFN-α, $p = 0.026$ for IFN-γ at 15 hr,) between control and stimulation. The dashed thick blue bar is for the mean, the thin black bar for the median.

Appendix A - Determination of internal control for gene expression studies in equine tissues and cell culture using quantitative RT-PCR

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Abstract

Quantitative reverse transcription polymerase chain reaction (RT-PCR) has become a basic, reliable and sensitive modern technique, in both biological research and clinical diagnosis, for investigation of gene expression and validation of cDNA microarray analysis. Accurate mRNA quantification using quantitative RT-PCR commonly requires data normalization through stable housekeeping genes (HKGs). Selection of HKGs for data normalization is critical for accurate mRNA quantification. Our objective was to evaluate a set of candidate HKGs as internal controls for gene expression studies using quantitative RT-PCR in equine tissues and cell culture. One-step quantitative RT-PCR for 6 HKGs was performed using total RNA from horse tissues and cultured peripheral blood mononuclear cells (PBMCs). The stability of HKGs was mainly evaluated by analysis of variance, analyses of the standard deviation and coefficient of variation of Ct, and change of Ct of HKGs between control and treated samples. 18S rRNA consistently showed the smallest standard deviation and coefficient of variation, and the least change of Ct between control and treated samples, thus was the most stable HKG for mRNA data normalization in quantitative RT-PCR for studying gene expression in equine tissues and cultured PBMCs.

Key words: Equine, housekeeping gene, quantitative RT-PCR, normalization

1. Introduction

Quantitative RT-PCR has become a reliable and sensitive contemporary approach for investigation of gene expression (Bustin, 2000; Bustin, 2002; Pusterla, Madigan et al., 2006; Draghici, Khatri et al., 2006; Draghici, Khatri et al., 2006). It can be performed in a one-step or two-step procedure. In two-step RT-PCR, the RT and PCR steps are carried out in the separate reactions with random hexamers, oligo(dT) or gene-specific primers for RT and gene-specific primers for PCR. In one-step RT-PCR, the RT and PCR steps are carried out in the same reaction with gene-specific primers. Compared to two-step RT-PCR, one-step RT-PCR has less chance for contamination and may provide higher sensitivity with optimized reaction (Wacker & Godard, 2005; Peters, Helps et al., 2004). In real-time PCR, gene expression can be monitored through fluorescence labeled gene-specific TaqMan probes or similar probes in a monoplex or multiplex reaction, or through SYBR Green I (a double-stranded DNA binding dye for monoplex reactions) with a melting-curve analysis to confirm the specificity of PCR reaction (Gomes-Ruiz, Nascimento et al., 2006; Bustin, 2000; Bustin, 2002). SYBR Green I has an economical advantage over TaqMan probes. In a monoplex reaction, both SYBR Green I and TaqMan probe-based real-time PCR assays are equivalent in sensitivity and specificity.

Quantitative RT-PCR data can be analyzed using an absolute standard curve method or relatively quantification method (Livak & Schmittgen, 2001; Pfaffl, 2001; Bustin, 2000). Absolute quantification determines the exact transcription copy number based on calibrated standard curves, which are labor intensive and time consuming. Relative quantification is often preferred because it only requires threshold cycle (Ct) numbers and estimated PCR amplification efficiencies of both target and housekeeping gene (HKG) in control and treated samples. The relative fold change of gene expression is usually normalized through a HKG in case of minor difference exists in starting amount of RNA, RNA quality, RT and PCR amplification efficiencies. The accurate estimation of PCR amplification efficiency also has a tremendous impact on mRNA quantification (Cikos, Bukovska et al., 2007). Thus, the challenge for accurate relative mRNA quantification through real-time RT-PCR is to find a stable HKG for data normalization.

The criteria for selecting a HKG for data normalization include that the HKG's mRNA expression is stable and minimally regulated under any experiment condition. However, there is

no universal HKG reported that can be applied for normalization of gene expression under all conditions. Thus, it is essential to find one or two best HKGs that are appropriate for data normalization under certain experimental conditions. Among the most commonly used HKGs are 18S rRNA (18S), Beta-actin (B-actin), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and hypoxanthine ribosyl transferase 1 (HPRT1).

In equine, there was no reported research on internal controls for studying gene expression in tissues and cultured cells using quantitative RT-PCR, except one involving only equine sarcoids (Bogaert, Van et al., 2006). Our objective was to apply some simple but unbiased statistical methods to evaluate a set of candidate HKGs as internal controls for gene expression studies in equine tissues and cell culture using SYBR Green I-based one-step quantitative RT-PCR.

2. Materials and Methods

2.1. Tissue sampling, cell culture and RNA isolation

All animal protocols were approved by the Institutional Animal Care and Use Committee at Kansas State University. Horse tissues were collected immediately postmortem from three euthanized adult Quarter Horses. Tissue samples for RNA isolation were snap frozen in liquid nitrogen and stored at -80 ^{o}C for further total RNA isolation. Samples collected included colon, heart, kidney, liver, lung, lymph node, small intestine and spleen. Whole blood samples were collected from 6 healthy, adult horses that were 3-25 years of age and appropriately vaccinated annually against West Nile virus (WNV). PBMCs from 6 adult Quarter Horses were isolated as previously reported by density gradient (Zhang, Davis et al., 2008). PBMCs (1 x 10^{6}/ml) were cultured in complete media (Zhang, Davis et al., 2008) at 37 ^{o}C, with or without purified WNV antigen (pre-membrane plus envelope proteins, 20 ug/ml, Fort Dodge Animal Health, Fort Dodge, IA) for 3-4 days before harvest for RNA extraction. Total RNA from tissue samples and cultured PBMCs was isolated and quantified as reported (Zhang, Davis et al., 2008). The total RNA quality was confirmed using RNA 6000 Nano Assay Kit (Agilent Technologies, Santa Clara, CA).

2.2. Quantitative RT-PCR

Primers for HKGs including 18S, 28S rRNA (28S), Beta2-microglobulin (B2M), B-actin, GAPDH and HPRT1 were designed using Beacon Designer 4.0 (PREMIER Biosoft International, Palo Alto, CA) for SYBR Green I assay (Table A.1). The specificity of each primer pair was confirmed by sequencing amplicon if unique product was amplified based on electrophoresis in 1-2% agarose gel, as well as melting-curve analysis in real time RT-PCR.

One-step quantitative RT-PCR was performed in duplicate on DNase I-treated total RNA samples from individual animals using a QuantiFast SYBR Green RT-PCR Kit (Qiagen Inc, Valencia, CA). In a Bio-Rad iCycler iQ Real Time PCR System (Bio-Rad, Hercules, CA), quantitative RT-PCR for these HKGs was performed in 15 ul reaction with 15 ng of total RNA and 0.6 uM final primer concentration for each forward and reverse primer. The RT-PCR protocol was 1 min annealing at the melting temperature of the reverse primer to ensure the specificity of RT reaction, 10 min RT reaction at 50 ^{o}C, 5 min PCR activation at 95 ^{o}C, then 40 cycles of 10 sec denaturation at 95 ^{o}C, 15 sec annealing at the lower of primer pair's melting temperature and 10 to 20 sec (depending on the length of amplicon) PCR extension at 72 ^{o}C, and finally a melting-curve analysis for confirming the RT-PCR specificity. Negative controls, without RT or total RNA, were included in each run of RT-PCR. Ct was automatically determined by the Optical System Software (Version 3.1) with the Bio-Rad iCycler iQ Real Time PCR System. The PCR amplification efficiency of each reaction was estimated by linear regression, in which 3 to 6 points covering the Ct value were utilized based on the highest correlation coefficiency (Ramakers, Ruijter et al., 2003).

2.3. Statistical Analyses

The data of each biological sample's duplicates were averaged. The Ct values, the key products of quantitative RT-PCR that have a negative correlation with the logarithm of transcription copy number of targeted genes, are crucial for accurate mRNA quantification. Thus, the stability of HKGs was mainly evaluated by analyzing the standard deviation and coefficient of variation of Ct, and change of Ct between control and treated samples.

Expression data were normalized as reported, using averaged amplification efficiencies of target and internal control genes (Pfaffl, 2001).

One way and two way analysis of variance or Kruskal-Wallis one way analysis of variance on ranks were performed in SigmaStat 3.5, with *p*-value less than 0.05 as significant. All pairwise multiple comparisons were performed with Holm-Sidak method.

3. Results and Discussion

Based the criteria for HKGs, we analyzed Ct values of 18S, 28S, B2M, B-actin, GAPDH and HPRT1 among horse tissues and cultured PBMCs. Among colon, heart, kidney, liver, lung, lymph node, small intestine and spleen, no significant difference was observed for 18S, 28S, B-actin, GAPDH and HPRT1 (Fig. A. 1). However, there was statistically significant ($p = 0.001$) difference in B2M between heart and colon, liver, lymph node, small intestine or spleen, thus B2M is the least stable HKG for normalization of tissue mRNA data. The significant higher Ct in B2M in the heart than colon, liver, lymph node, small intestine and spleen suggests a lower expression of major histocompatibility complex (MHC) class I antigens. This is consistent with what was observed in normal healthy cardiac tissue unless inflammation occurs due to disease or heart transplantation rejection in humans (Zavazava, Bottcher et al., 1993; Hengstenberg, Hufnagel et al., 1993).

WNV antigen stimulates substantial *in vitro* antigen-specific CD4 & CD8 T cell proliferation due to vaccination and/or natural exposure to WNV in adult horses (data not shown). The expression of 18S, 28S, B2M, B-actin, GAPDH and HPRT1 was investigated in PBMCs cultured with or without purified WNV antigen. No significant difference in Ct of these 6 HKGs between control and stimulated PBMCs was observed (Fig. A. 2). For cultured PBMCs, the expected ΔCt (difference in Ct of a HKG between control and stimulated PBMCs) should be near 0, indicating a good stability of the HKG (Pfaffl, 2001). However, the data (Fig. A. 2) showed none of the 6 HKGs met the expectation even though equal amount of total RNA was applied for each RT-PCR reaction, indicating possible uneven efficiencies in RT and/or PCR steps.

Among tissues or cultured PBMCs, no statistically significant difference observed in HKGs' Ct does not decisively indicate these HKGs are equivalent for mRNA data normalization. In cultured PBMCs, the ΔCt (difference in Ct between control and stimulated PBMCs) was analyzed (Fig. A. 2). 18S and 28S showed the least change in Ct between control and stimulated PBMCs, suggesting they might be the appropriate HKGs for mRNA data normalization. We further analyzed the Ct values across all tissues and between cell culture conditions. Compared to the other 5 HKGs, 18S consistently showed the smallest standard deviation and coefficient of variation even though 18S had the lowest Ct values (Table A.2). These results demonstrated that 18S is the most stable HKG in quantitative RT-PCR for mRNA data normalization.

PCR amplification efficiencies of the 6 HKGs for all quantitative RT-PCR reactions were estimated by a linear regression method (Ramakers, Ruijter et al., 2003). These estimates were very high and also showed high consistency or stability across samples (106.46% ± 5.61% (mean ± standard deviation) for 18S, 120.17% ± 9.68% for 28S, 100.00% ± 8.57% for B2M, 99.24% ± 9.13% for B-actin, 103.31% ± 7.88% for GAPDH and 99.47 % ± 10.16% for HPRT1). No statistically significant difference was observed in PCR amplification efficiencies of the 6 HKGs across all tissues and between cell culture conditions.

The mRNA data from quantitative RT-PCR are normalized to HKGs to justify minor differences in starting amount of RNA, RNA quality, RT and PCR amplification efficiencies, thus accurate relative gene expression data could be obtained. However, there is no universal HKG found as a stable internal control for mRNA data normalization. Although researchers have spent a tremendous amount of effort to develop a reasonable method to search stable HKGs or complicated algorithm for normalizing mRNA data from quantitative RT-PCR, there is no perfect method that has been reported. A recent report regarding validation of candidate bovine reference genes with real-time PCR applied three computer programs (BestKeeper, NormFinder and geNorm) that unanimously identified B-actin and GAPDH as the most appropriate HKGs in blood for data normalization (Robinson, Sutherland et al., 2007). Interestingly, this could simply be achieved when the standard deviation and coefficient of variation of HKGs' Ct values were ranked. Such a simple but practical method appeared to have enough power for the search of stable HKGs for data normalization. Thus we applied this efficient simple method for our study and identified that 18S is the most appropriate HKG in quantitative RT-PCR for mRNA data normalization in equine tissues and cultured PBMCs.

As shown in our experiment, 18S is the most suitable HKG for mRNA expression normalization because it shows the highest stability as a HKG. However, many HKGs after stimulation (Fig. A. 2) or 18S in tumor cells (Tricarico, Pinzani et al., 2002) might be slightly upregulated. Thus, to find a suitable HKG for mRNA expression normalization appeared to be essential for every experimental condition.

Many factors could contribute to the variation in HKGs' Ct estimates. In our experiment, no significant correlation between Ct and PCR amplification efficiencies was observed for 18S, B2M, B-actin, GAPDH and HPRT1, indicating that PCR amplification efficiency was not a factor contributing the Ct variation of these 5 HKGs. Sample variation could contribute most even though equal amount total RNA was applied. Other factors like the relative uncertainty of DNA amplification due to inherent stochastic kinetics of RT-PCR reaction (Peccoud & Jacob, 1996), the operators and time could also account for the variation of Ct estimates (Bustin, 2002). Such artificially and systemically introduced variation in Ct estimates in quantitative RT-PCR is thus exponentially amplified after data normalization in relative quantification of mRNA. This brings up two questions: i) Can we solve the circular problem of finding a stable HKG for data normalization even though there appears to be no reliable method to evaluate the stability of candidate HKGs? (Andersen, Jensen et al., 2004); ii) Which data, Ct or normalized mRNA data, should be analyzed? There is no doubt that real time or quantitative RT-PCR is an accurate and highly sensitive modern technique in mRNA quantification. However, it requires highly specialized expertise and techniques to ensure the accuracy and precision of data acquisition and thus better opportunity to find a reliable HKG for data normalization. Analysis of Ct data for the determination of HKGs' stability under experimental conditions is nonetheless a good approach and independent of the targeted genes. For targeted genes, the analysis of variance in normalized mRNA data instead of Ct data of target genes statistically requires more biological replicates to ensure significant results due to the exponential amplification of artificially and systemically introduced variation. If no reliable HKG for data normalization can be identified, Ct-based analysis of relative target gene expression is a good choice as long as total RNA for each RT-PCR reaction is equally loaded, the quantitative RT-PCR reactions for each target or reference gene are performed simultaneously and the result presents meaningful biological significance. The relative fold change in gene expression can be computed by $(1+E)^{\Delta Ct}$, where E is the average PCR amplification efficiency of the target gene. This approach is equivalent as considering ΔCt

of the reference HKG as 0 (Pfaffl, 2001). It can also be considered as normalizing the target gene expression to equally loaded total RNA. Equal loading of total RNA in quantitative RT-PCR is the foundation of evaluating internal controls' stability. This approach has an advantage of avoiding the introduction of external variation in mRNA quantification due to normalization against a possibly fallible HKG, but has a disadvantage of not justifying the unavoidable error from the measurement of sample RNA concentration.

Acknowledgments

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References

Andersen, C. L., Jensen, J. L., Orntoft, T. F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64, 5245-5250.

Bogaert, L., Van, P. M., De, B. C., Peelman, L., Gasthuys, F., Martens, A., 2006. Selection of a set of reliable reference genes for quantitative real-time PCR in normal equine skin and in equine sarcoids. BMC. Biotechnol. 6, 24.

Bustin, S. A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 25, 169-193.

Bustin, S. A., 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. 29, 23-39.

Cikos, S., Bukovska, A., Koppel, J., 2007. Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. BMC. Mol. Biol. 8, 113.

Draghici, S., Khatri, P., Eklund, A. C., Szallasi, Z., 2006. Reliability and reproducibility issues in DNA microarray measurements. Trends Genet. 22, 101-109.

Gomes-Ruiz, A. C., Nascimento, R. T., de Paula, S. O., Lopes da Fonseca, B. A., 2006. SYBR green and TaqMan real-time PCR assays are equivalent for the diagnosis of dengue virus type 3 infections. J. Med. Virol. 78, 760-763.

Hengstenberg, C., Hufnagel, G., Haverich, A., Olsen, E. G., Maisch, B., 1993. De novo expression of MHC class I and class II antigens on endomyocardial biopsies from patients with inflammatory heart disease and rejection following heart transplantation. Eur. Heart J. 14, 758-763.

Livak, K. J., Schmittgen, T. D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25, 402-408.

Peccoud, J., Jacob, C., 1996. Theoretical uncertainty of measurements using quantitative polymerase chain reaction. Biophys. J. 71, 101-108.

Peters, I. R., Helps, C. R., Hall, E. J., Day, M. J., 2004. Real-time RT-PCR: considerations for efficient and sensitive assay design. J. Immunol. Methods 286, 203-217.

Pfaffl, M. W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Pusterla, N., Madigan, J. E., Leutenegger, C. M., 2006. Real-time polymerase chain reaction: a novel molecular diagnostic tool for equine infectious diseases. J. Vet. Intern. Med. 20, 3-12.

Ramakers, C., Ruijter, J. M., Deprez, R. H., Moorman, A. F., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62-66.

Robinson, T. L., Sutherland, I. A., Sutherland, J., 2007. Validation of candidate bovine reference genes for use with real-time PCR. Vet. Immunol. Immunopathol. 115, 160-165.

Tricarico, C., Pinzani, P., Bianchi, S., Paglierani, M., Distante, V., Pazzagli, M., Bustin, S. A., Orlando, C., 2002. Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal. Biochem. 309, 293-300.

Wacker, M. J., Godard, M. P., 2005. Analysis of one-step and two-step real-time RT-PCR using SuperScript III. J. Biomol. Tech. 16, 266-271.

Zavazava, N., Bottcher, H., Ruchholtz, W. M., 1993. Soluble MHC class I antigens (sHLA) and anti-HLA antibodies in heart and kidney allograft recipients. Tissue Antigens 42, 20-26.

Zhang, Y. W., Davis, E. G., Blecha, F., Wilkerson, M. J., 2008. Molecular cloning and characterization of equine Toll-like receptor 9. Vet. Immunol. Immunopathol. 34, 209-219.

Table A.1. Housekeeping gene primers for quantitative RT-PCR

Gene	GenBank accession No.	Forward Primer (5'~3')	Reverse Primer (5'~3')	Amplicon Length (bp)
18S	AJ311673	ATGCGGCGGCGTTATTCC	GCTATCAATCTGTCAATCCTGTCC	204
28S	EU554425	CGGGTAAACGGCGGGAGTAAC	TAGGTAGGGACAGTGGGAATCTCG	109
B-actin	AF035774	CACCACACCTTCTACAAC	ATCTGGGTCATCTTCTCG	107
B2M	X69083	GGCTACTCTCCCTGACTGG	ACACGGCAACTATACTCATCC	271
GAPDH	AF157626	GCCATCACCATCTTCCAG	GACTCCACAACATATTCAGC	77
HPRT1	AY372182	GAGATGTGATGAAGGAGATG	TGACCAAGGAAAGCAAGG	300

Table A.2. Descriptive statistics of threshold cycles (Ct) of 6 housekeeping genes in horse tissues and cultured PBMCs, where PBMCs were cultured for 3 days, with or without stimulation of West Nile virus antigen before being harvest for total RNA isolation. Stdev is for standard deviation of Ct. CV is for coefficient of variation.

Gene	Tissues (n=24)			Cell culture (n=12)		
	Ct Mean	Stdev	CV	Ct Mean	Stdev	CV
18S	11.9	0.43	3.56%	11.6	0.49	4.20%
28S	10.1	0.55	5.46%	9.8	0.62	6.30%
B2M	18.5	1.62	8.73%	15.4	0.73	4.75%
B-actin	15.5	0.73	4.73%	16.4	1.08	6.63%
GAPDH	18.8	0.82	4.38%	18.7	0.84	4.53%
HPRT1	24.4	1.41	5.78%	22.0	0.79	3.61%

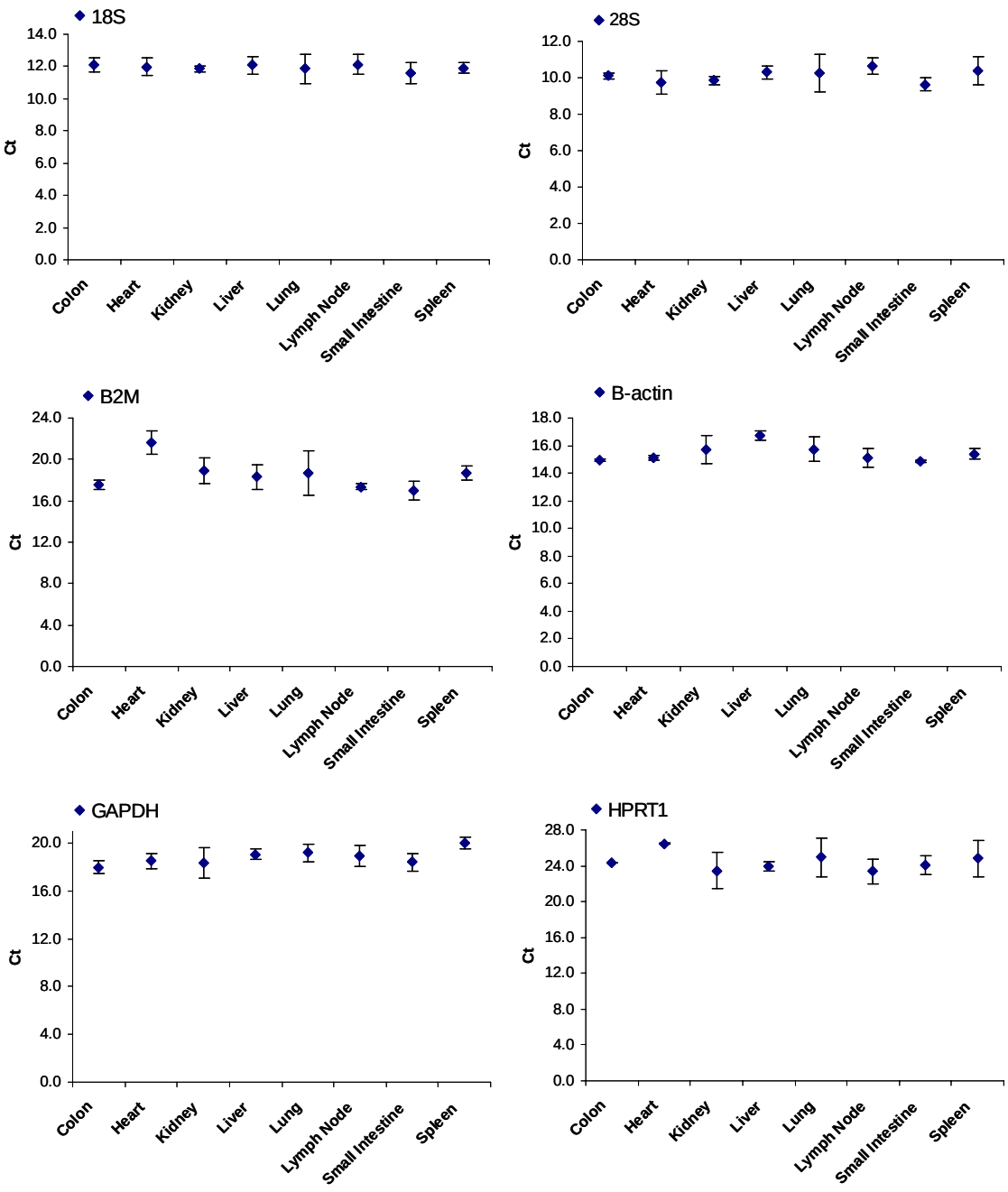

Fig. A.1. Comparison of threshold cycle (Ct) of 6 housekeeping genes for quantitative RT-PCR among 8 horse tissues. Pairwise comparison showed statistically significant difference in B2M between heart and any of colon, liver, lymph node, small intestine and spleen. The error bar shows 95% confidence interval.

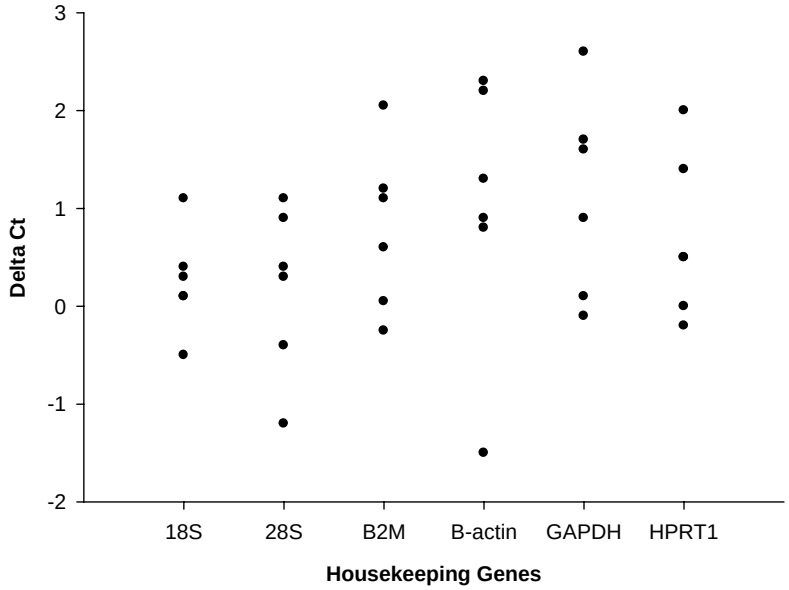

Fig. A.2. Relative expression of 6 housekeeping genes in PBMCs after culture for 3 days, with or without stimulation of West Nile virus antigen. Delta Ct is the Ct difference between control and stimulated PBMCs.