Development of Hyperimmune Equine Plasma to Virulence Associated Protein A (VapA) of *Rhodococcus equi*

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Objective – To develop a new potent *Rhodococcus equi* vaccine for use on equine plasma donors. To determine efficacy of equine plasma in a clinical trial. To develop a quantitative assay to determine potency of plasma derived from vaccinated donors.

Procedure – A proprietary formulation of ATCC 33701 *Rhodococcus equi* known to contain the virulence plasmid was developed and tested on 72 horses. Antibody levels to *Rhodococcus equi* Virulence associated protein A (VapA) were determined relative to a Standard Plasma. A clinical trial was performed on 28 foals. Seven foals were administered hyperimmune plasma and seven foals were administered purified equine immunoglobulin specific for VapA and VapC one day prior to intrabronchial challenge with *Rhodococcus equi* strain 103+. Eleven foals were challenged with no prior treatment and three foals received no challenge or treatment. A quantitative anti-VapA antibody enzyme immunoassay (EIA) was developed by using known concentrations of purified anti-VapA antibodies to create a standard curve.

Results – The new ATCC 33701 *Rhodococcus equi* vaccine has doubled potency towards VapA in the donor herd. Treatment with hyperimmune plasma or immunoglobulins specific to VapA and VapC significantly delayed the onset of clinical signs of pneumonia, decreased lung lesion severity and reduced the number of *Rhodococcus equi* organisms in lungs of challenged foals. A quantitative EIA to VapA has been developed. An efficacious Standard Plasma has a concentration of 400 µg/mL of anti-VapA antibody. Immunoglobulins in plasma are very stable and tolerant of long periods of frozen storage and multiple freeze / thaw cycles.

Conclusions and Clinical Relevance – Plasvacc has demonstrated efficacy of its Equiplas R products in a clinical trial. Plasma used in this clinical trial had a concentration of anti-VapA antibodies of 400 µg/mL when measured using a quantitative EIA. Despite the demonstrable stability of immunoglobulins, Plasvacc recommends strict adherence to label instructions with respect to storage and use of equine plasma.

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Introduction

Rhodococcus equi is an important pathogen of young foals for which there are few treatments. The organism is ubiquitous and a normal intestinal inhabitant of horses. As such, all foals are exposed early in life, and normal adults show some immunological response to this organism[1]. However, some foals develop disease and others do not. Some of the factors that are considered important in whether a foal develops disease include:

- The virulence of the strain of *Rhodococcus equi* found endemically on a farm and whether it contains a virulence plasmid expressing Virulence Associated Proteins (Vap),
- Level of exposure and challenge dose[2],
- Ambient temperature, soil pH, soil type,
- Dusty conditions[3],[4]
- Development of an appropriate immune response[5].

Husbandry procedures can be implemented on farms to control exposure of young foals to virulent and endemic *Rhodococcus equi* and to detect the disease as early as possible. However, because foals and not adults are susceptible to the disease the development of an appropriate immune response in foals has been the focus of much research including the development of preventions and therapies.

While cellular immune responses are postulated to be critical to clearance of *Rhodococcus equi*, due to the bacterium's status as a facultative intracellular pathogen, there is evidence that both antibody and T lymphocytes play a role in immunity and that secretion of appropriate cytokines is required[6]. By example:

- Humoral immunity seems to be critically involved in the early protection of young foals[7],
- The enhancing effect of sera from foals with R. equi infection on macrophage opsonic activity of the organism has been demonstrated[8],
- Mares vaccinated with *Rhodococcus equi* Virulence Associated Protein (VapA) have higher IgG and opsonic activity that is transferable to foals[9] - such opsonic activity may assist the cellular immune system in clearance of the bacteria,
- Various *Rhodococcus equi* antigens, including the plasmid-encoded virulence-associated proteins, the exoenzymes known as 'equi factors', and capsular polysaccharides, have been postulated to induce a protective response[10], in part due to the induction of an appropriate antibody response.

The susceptibility of foals less than 4-6 months of age is postulated to reflect waning maternal antibody, and passive transfer of hyperimmune plasma can provide protection on endemic farms[11]. Giguere and Jacks (2005) performed a meta-analysis of published clinical and research studies of the effectiveness of hyperimmune plasma revealing a significant overall positive effect with odds ratio of a positive outcome of 4.6 (95% confidence interval of 1.1 – 19; $p<0.05$)[12]. Examples of such studies are referenced here[13],[14],[15],[16].

Once foals have been identified to be affected by *Rhodococcus equi* treatment options are limited. The following paragraph from the Ontario Ministry of Agriculture, Food and Rural Affairs[17] succinctly describes current approaches to the treatment of *Rhodococcus equi* in foals –

"Affected foals need prolonged treatment because of the persistence of the bacteria within abscesses in the lung and because immunity to lung infection is poor, the disease tends to recur. Recent research reveals the combination of the antibiotics, erythromycin and rifampin is effective in the treatment of the disease. These expensive drugs can be given orally, a definite advantage over alternative treatments. These drugs penetrate the phagocytic cells (the cells which ingest foreign material) where *Rhodococcus equi* are found, and are not toxic when used over prolonged periods. The combination should be used for at least one week past cure, determined clinically by the use of x-rays or by blood tests for normal fibrinogen levels. Other drugs, such as gentamicin or trimethoprim-sulfamethoxazole, are effective but need to be injected. Because of its toxicity, gentamicin cannot be used for prolonged periods in foals suffering from this disease. However, controlling the infection on endemic farms solely by treating diseased foals is both an ineffective and expensive approach."

More recent research conducted by Giguere and Jacks (2005)[18] concluded that although combined therapy with erythromycin and rifampin has dramatically improved the survival rate of foals infected with *Rhodococcus equi*, this treatment regimen is not without problems. Erythromycin has poor and variable oral bio-

availability in foals, requires multiple daily dosing, and most importantly, has a high incidence of adverse effects. The combination clarithromycin-rifampin was found to be more effective than erythromycin-rifampin or azithromycin-rifampin especially in foals with severe radiographic lesions. Foals treated with clarithromycin had a higher incidence of diarrhoea than those treated with azithromycin. In most cases, diarrhoea was mild and self-limiting.

Therefore antibiotic treatment of *Rhodococcus equi* infection in foals can be effective but it is expensive, not without side effects, and not recommended as a sole approach in managing the disease.

The administration of equine plasma rich in antibodies to *Rhodococcus equi* to foals, and in particular plasma containing antibodies to virulence associated proteins, can be considered to be beneficial to foals whilst potentially avoiding the use of expensive antibiotics that have adverse side effects.

To this end, Plasvacc has conducted unique and extensive work towards the development of equine plasma with high levels of specific antibodies to the Virulence Associated Protein A (VapA) of *Rhodococcus equi* including:

- An effective stimulatory VapA vaccine,
- A specific quantitative VapA antibody potency assay,
- A clinical efficacy trial,
- Stability studies,
- USDA registration,
- Production under Good Manufacturing Principles (GMP).

Such work aims to provide the equine veterinarian with professional confidence in the knowledge they are using an effective, potent, appropriately labelled, and safe product.

This white paper presents detail on the development of Plasvacc Equiplas R and REA®.

Materials and Methods

Vaccine

Plasvacc has developed a new proprietary vaccine formulation of Amercian Type Culture Collection (ATCC) 33701 *Rhodococcus equi*. This strain has known expression of VapA and was originally isolated from a clinical case by University of Guelph[19]. Horses were immunised with multiple doses of vaccine and increasing titres of anti-VapA antibody were monitored by Enzyme Immunoassay (EIA) and results expressed relative to a Standard Reference Plasma (1.00).

Quantitative Potency Assay (EIA)

A quantitative EIA was developed to measure specific antibodies to VapA in equine plasma. In brief, anti-VapA antibodies (AVA) were purified from multiple equine plasmas of known status using affinity chromatography. An accurate concentration of an AVA preparation (mg/mL) was determined using both Nanodrop[20] and BCA[21] techniques. Known quantities of AVA were then used in an indirect EIA to generate a standard curve. Reagents used in the EIA included recombinant VapA as the antigen bound to the plate and goat anti-horse IgG (H+L) conjugated to Horse Radish Peroxidase (HRP) as the reporter molecule. Concentration of AVA (µg/mL) was then determined in equine plasma of unknown status by comparison to the standard curve.

Efficacy

A clinical trial in foals was performed to determine efficacy of hyperimmune plasma specific for *Rhodococcus equi*[22]. In brief, seven foals were administered hyperimmune plasma and seven foals were administered purified equine immunoglobulin specific for VapA and VapC one day prior to intrabronchial challenge with *Rhodococcus equi* strain 103+. Eleven foals were challenged with no prior treatment and three foals received no challenge or treatment. Clinical and blood parameters were determined daily. Lung lesions at Day 14 post-challenge were assessed at autopsy.

Stability

Various batches of equine plasma in PVC bags and in the same volume as sold product were tested for sterility, cell count, total protein, total immunoglobulin G (IgG) and specific anti-VapA antibody levels following being subjected to:

- Freezing (-22^{0}C) for up to 12 years,
- Refrigeration (4^{0}C) for up to 108 days,

- Freeze (-20^{0}C) / Thawing (22^{0}C) up to four times.

Sterility testing was performed according to the USA Code of Federal Regulations (9 CFR 113.26). Cell counts were performed using an Abbott Cell-dyn 3500R. Total protein was determined by refractometer. Total IgG was determined by Radial Immunodiffusion (RID). Anti-VapA antibodies were determined by comparison to a standard reference plasma using EIA.

USDA and GMP

Plasvacc Equiplas® R and REA are produced under quarantine conditions and GMP, and is licensed through the United States Department of Agriculture (USDA) for sale through veterinarians only.

All horses are tested for blood type and various diseases prior to entry to quarantined premises and on an on-going basis. Plasma is guaranteed to be: derived from healthy horses, sterile, free of disease, have minimal cell counts, and to meet potency standards.

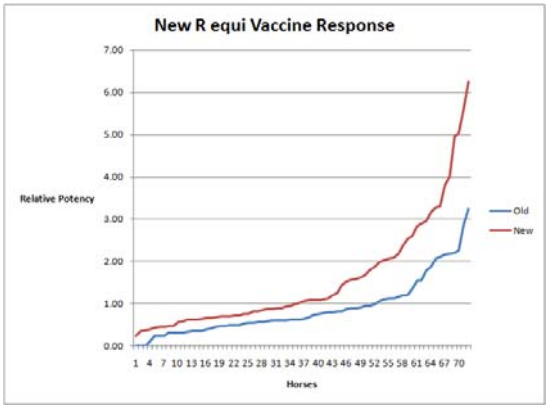
GMP licensing through the USDA means Plasvacc is regularly audited. A license can be suspended at any time if production standards are not met.

Results

Vaccine

Plasvacc's original vaccine consisted of a killed bacterin of multiple strains of *Rhodococcus equi* of unknown VapA expression. The new vaccine consists of a killed bacterin of proprietary formulation containing ATCC 33701 *Rhodococcus equi* known to express VapA. The VapA relative potency results following multiple doses of this vaccine on a herd of 72 horses that had received multiple doses of the old vaccine are shown in Figure 1.

Figure 1
The following graph plots plasma VapA relative potency for 72 horses when using the old (before) and new (after) Rhodococcus equi vaccines.

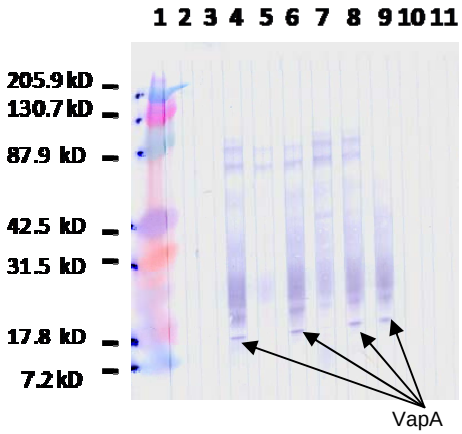


Using the old vaccine 20 of 72 horses had a relative VapA potency of at least 1.00 (note that Equiplas R is only ever released for sale if it reaches a Relative Potency of 1.00). Using the new vaccine 37 of 72 horses had a relative VapA potency of at least 1.00 and 19 horses have a relative potency of at least 2.00.

Quantitative Potency Assay (EIA)

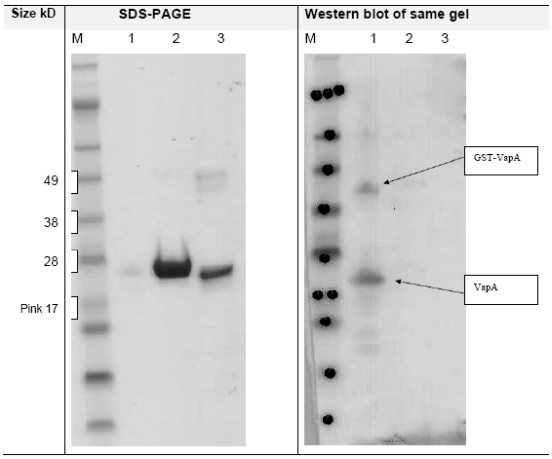
Anti-VapA antibodies for use as a standard in an EIA were purified from the plasma of horses vaccinated with the new *Rhodococcus equi* bacterin and known to produce antibodies to VapA as determined by western blot (see Fig. 2).

Figure 2
The following figure contains strip westerns performed on whole Rhodococcus equi blotted against a series of equine plasmas. Antibodies to VapA are arrowed (it is known that VapA has a molecular weight of 18kD). Only those plasmas known to have antibodies to VapA were used to generate purified anti-VapA antibodies.



The specificity of purified anti-VapA antibodies was tested by western blot (see Figure 3).

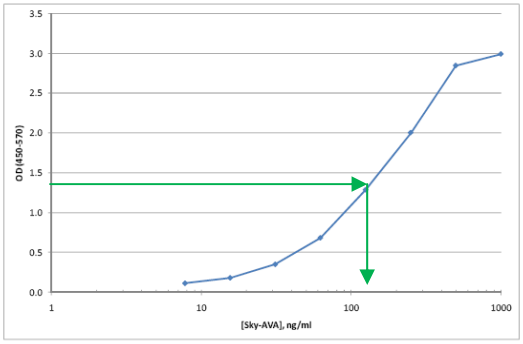
Figure 3
The following photographs are of an SDS-PAGE gel (left) and its corresponding western blot (right). The gel contains, from left to right in lanes 1, 2, 3, size marker, recombinant GST-VapA, recombinant GST and human placenta GST (Sigma). The western blot was probed with the purified anti-VapA antibody preparation and both GST-VapA and VapA (arrowed) react specifically with the purified anti-VapA antibody preparation and not GST alone.



It can be seen from the western blot that the purified anti-VapA antibody reacts only with VapA and GST-VapA (arrowed) and not with either recombinant GST or human placenta GST. The specificity of the purified anti-VapA preparation is important for accurate determination of anti-VapA levels using EIA.

The anti-VapA antibody was then quantified using two methods, BCA and Nanodrop. Each of these methods determined that the concentration of the anti-VapA standard was 270 µg/mL and 279 +/- 9 µg/mL respectively. Known concentrations of the anti-VapA standard were then used to create a standard curve (Figure 4).

Figure 4
The following figure plots optical density (OD, Y axis) obtained in an EIA for a series of known concentrations of anti-VapA antibodies (X-axis). If the OD of an unknown plasma is determined using the same EIA, the concentration of anti-VapA antibodies can be determined (green lines by example).



The concentration in µg/mL of anti-VapA antibody in unknown diluted plasma samples can be determined from OD (optical density) readings (see Figure 4). The concentration of anti-VapA antibodies in a Standard Plasma, demonstrated to be effective in a clinical trial, was 400 µg/mL.

Efficacy

The onset of clinical signs of pneumonia was significantly delayed in foals administered hyperimmune plasma and purified equine IgG specific for VapA and C compared to the control group. In addition, pulmonary lesions were less severe in the treated groups and significantly fewer *Rhodococcus equi* organisms were isolated from the lungs of treated foals.

Stability

Stability results from a single batch of Equiplas manufactured in 2005 and stored continuously at -20^{0}C are contained in Table 1.

Table 1

	Equiplas Batch 1548		
Year	IgG (mg/mL)	Cell Count	Sterility
2005	24	0	NG
2007	22	0	NG
2008	22	0	NG

NG: No growth. Cell counts are below detectable levels.

Following frozen storage it can be seen that Equiplas IgG levels have remained stable, cell counts have not changed and it has remained sterile.

Results from further stability work on a single batch of Equiplas R® stored continuously at -20^{0}C are contained in Table 2.

Table 2

	Equiplas Batch 4802			
Year	ELISA VapA (Log 10)	IgG (mg/mL)	Total Protein	Sterility
1997		16.7	5.2	NG
2002		17	5.2	NG
2006	3.600	18.9	5.4	
2007	3.500	18.5	5.4	NG
2008	3.500			
2009	3.700	20.5	5.6	NG

NG: No growth

Following frozen storage for 12 years it can be seen that specific antibody titres to VapA, and total IgG and protein have remained stable in Equiplas.

Results of potency measured relative to a Standard Plasma for two further batches of Equiplas R® subjected to multiple freeze/thaw cycles and refrigeration are contained in Table 3.

Table 3

Plasma #	Treatment	RP
1	Freeze / Thaw 1	1.31
1	Freeze / Thaw 2	1.32
1	Freeze / Thaw 3	1.36
2	Freeze / Thaw 1	1.47
2	Freeze / Thaw 4	1.37
2	Refrigeration 8 days	1.27

Following multiple freeze / thaw cycles (up to four) and refrigeration for 8 days the potency of antibodies to VapA relative to a Standard Plasma has remained stable.

Discussion

The scientific literature adequately demonstrates that *Rhodococcus equi* must possess a virulence plasmid producing virulence associated proteins (Vap) to be able to cause disease in foals[23]. The scientific literature also demonstrates that both cellular and humoral immunity play a role in host defence against this intracellular organism and that foals are most susceptible to the effects of virulent organisms when maternal antibody levels are waning. Further, the use of hyperimmune plasma has been demonstrated to have an overall positive effect on outcome. Plasvacc therefore aims to produce plasma from horses repeatedly exposed to virulent *Rhodococcus equi* with known potency to Virulence-associated protein A (VapA). To this end, Plasvacc has generated a proprietary virulent *Rhodococcus equi* vaccine for use on its donor horses.

The new vaccine is effective at generating an anti-VapA response in Plasvacc's horse herd, as measured relative to a Standard Plasma. Many more of Plasvacc's horses are producing plasma with an anti-VapA relative potency greater than 1.00. Plasma of this strength has been demonstrated in an efficacy trial to be sufficient to delay the onset of clinical signs and reduce the severity of lesions and reduce the number of *Rhodococcus equi* organisms.

Prior to the development of a quantitative potency assay Equiplas R® potency was determined by comparison to a Standard Plasma known to be efficacious. Equiplas R® plasma was released for sale if it had a relative potency, compared to the Standard Plasma, of at least 1.00. The accuracy of such a test relies in part on the stability of the Standard Plasma. Plasvacc have now developed the world's first quantitative EIA capable of determining the level, in µg/mL, of anti-VapA antibodies in equine plasma. Knowing the concentration of anti-VapA antibodies in an efficacious plasma in micrograms per millilitre allows for a more accurate determination of the potency of plasma released for sale. Veterinarians can now more easily benchmark the potency of the plasma they are using since it is known that plasma containing at least 400 µg/mL is effective.

Immunoglobulins are known to be very stable molecules in purified form or in plasma. Plasvacc has conducted studies over 12 years demonstrating that equine plasma is stable for long periods when stored at -20^{0}C or below, for up to 8 days at 4^{0}C and following multiple frreze / thaw cycles. Despite these findings, Plasvacc strongly recommends following the storage guidelines provided on the label and disposing of plasma that has expired.

Conclusion

Plasvacc has a new, highly effective and proprietary *Rhodococcus equi* vaccine for use in generating equine plasma with high levels of anti-VapA antibody. Further, Plasvacc has developed the world's first quantitative anti-VapA assay for determining the quantity of anti-VapA antibodies in equine plasma in micrograms per millilitre.

These advances, along with Plasvacc's continued quality assurance and product registration, ensure that equine veterinarians are provided with high quality product of known potency.

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