

**EXPRESSION OF VIRULENCE-ASSOCIATED PROTEIN A (VapA)
OF *RHODOCOCCLUS EQUI* BY INFECTED MACROPHAGES**

A Senior Scholars Thesis

by

KAYTEE BETH WEAVER

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

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Approved by:

Research Advisor:
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ABSTRACT

Expression of Virulence-Associated Protein A (VapA) of *Rhodococcus equi* by Infected Macrophages. (April 2011)

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Rhodococcus equi is a Gram-positive bacterium known to cause disease because of its ability to survive and replicate inside macrophages. Macrophages also act as antigen-presenting cells: peptides from bacteria that have been engulfed and processed by macrophages are presented on the external surface of the macrophages to immune cells, primarily lymphocytes. Virulent *R. equi* contain an 85-kb plasmid that encodes for a virulence-associated protein A (VapA) that has been shown to be required for clinical disease in foals. It is unknown whether macrophages infected with virulent *R. equi* express VapA peptides on their surface. Thus, the objective of this experiment was to infect J774.A1 murine macrophages with virulent and avirulent strains of *R. equi* and to determine whether VapA was expressed on the surface of the infected macrophages. The macrophages were first infected with a virulent strain of *R. equi* that were labeled with a fluorochrome (pHrodo™; Invitrogen, Carlsbad, CA, USA), and tested using flow cytometry to verify that phagocytosis of *R. equi* by the macrophages occurred efficiently. After confirming that phagocytosis occurred efficiently and consistently, macrophages

were co-cultured with the isogenic virulent and avirulent *R. equi*. Macrophages infected with virulent but not avirulent strains of *R. equi* expressed VapA on their surface.

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CHAPTER I

INTRODUCTION

Rhodococcus equi is a Gram-positive bacterium found ubiquitously in the environment, and is the leading cause of severe pneumonia in young foals (6). Virulent strains of *R. equi* are characterized by possession of an 85-kb plasmid which encodes the virulence-associated protein (VapA). VapA is expressed on the surface of virulent isolates of *R. equi*, and it is considered to be an immunodominant protein (2). *Rhodococcus equi* is a facultative intracellular pathogen that is capable of replicating and surviving within macrophages (3). Although a substantial amount of research has focused on the interactions between *R. equi* and macrophages, there is a paucity of information regarding processing and presentation of VapA peptides by infected macrophages. Peptide antigens are expressed on the surface of antigen-presenting cells (APCs, e.g., macrophages) through major histocompatibility complex (MHC) class I or II molecules. MHC class I molecules present endogenously-derived peptides (e.g., intracellular bacterial pathogens) to cluster of differentiation (CD)-8-positive cytotoxic T lymphocytes (CTL's), while MHC class II molecules present exogenously derived peptides (e.g., extracellular bacterial pathogens) to CD-4-positive (CD-4+) T helper (Th) 1 or Th2 lymphocytes (5). It has been demonstrated that CD-4+ Th cells and CD-8+ CTL's play an important role in the clearance of *R. equi*. CD-8+ T lymphocytes and CD-4+ Th cells contribute not only in secreting interferon gamma (IFN- γ), but CD-8+ CTL's

This thesis follows the style of *Infection and Immunity*.

recognize and lyse infected host cells (6).

Macrophages function in the immune response by phagocytosing pathogens and subsequently killing them. *Rhodococcus equi* has been proved threatening in its ability to replicate within macrophages after being engulfed. (3). *Rhodococcus equi* can be ingested by macrophages following binding of *R. equi* to the Mac-1 receptor (a.k.a. complement receptor 3 [CR3]) when not opsonized by antibodies, and through the Fc γ RIII receptor when the bacteria are opsonized by antibodies (4). It appears that *R. equi* taken into macrophages by the Mac-1 receptor can survive intracellularly, by preventing phagosome-lysosome fusion and inhibiting acidification of the phagolysosome (4, 10). Because the fate of virulent *R. equi* taken up by macrophages may vary, it is unclear to what extent there is processing of bacterial peptides for expression on the cell surface of macrophages. To our knowledge, it remains unknown whether macrophages infected with virulent *R. equi* have evidence of VapA peptides presented on their surface. The purpose of this study was to determine whether a monoclonal antibody against VapA would bind to the cell surface of macrophages infected with virulent *R. equi* but not to macrophages infected with avirulent *R. equi*. These data would provide evidence of antigen presentation by infected macrophages, an important step in cell-mediated immune recognition.

CHAPTER II

METHODS

Bacteria

R. equi ATCC 33701+ (a virulent strain producing VapA that was originally isolated from an infected foal) and ATCC 33701- (an isogenic strain lacking the virulence-associated plasmid which encodes VapA), were streaked on brain heart infusion (BHI) agar plates and incubated for 48 h at 37°C. Isolated colonies were collected and shaken in BHI broth for 24 h at 37°C. The bacteria were washed 3 times with phosphate buffered saline (PBS) and diluted to an optical density of 1. For 1 trial, the bacteria were opsonized with 10% diluted equine serum (50% in PBS) for 30 min at 37°C with constant rotation before infection.

Macrophage cell culture

J774.A1 macrophages were obtained from the laboratory of Dr. Thomas Ficht, Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University. The macrophages were cultured in 1-X Dulbecco's modified Eagle medium, DMEM (Gibco-BRL, Grand Island, NY, USA), with 10% fetal bovine serum, FBS (Gibco), and 1% non-essential amino acids, NEAA (Gibco), at 37°C with 5% CO₂. In preparation for infection followed by 24 h gentamicin incubation to remove extracellular bacteria, 7.5 x10⁵ cells/well were placed in 6-well plates. The plates were incubated for 24 h to allow for the macrophages to adhere.

Bacterial phagocytosis by macrophages using flow cytometry

To ensure the macrophages were taking up the *R. equi*, pHrodo™ phagocytosis labeling particles (Invitrogen) were used to label virulent *R. equi* (5mg/ml). The labeled *R. equi* were opsonized with 10% of diluted serum in PBS as previously mentioned and introduced to the macrophages (multiplicity of infection of 30:1 bacteria: macrophage) for 30 min at 37°C with constant rotation. Macrophages infected with labeled *R. equi* incubated at 4°C served as negative controls along with non-infected cells incubated at 4°C and 37°C. After incubation, all the cells were placed on ice for 5 min to stop phagocytosis. The tubes were centrifuged at $350 \times g$ for 5 min, supernatants removed and resuspended in PBS for analysis by flow cytometry.

Bacterial infection of macrophages

Plated macrophages were infected with strains 33701+ and 33701- at a multiplicity of infection of 5:1 bacteria: macrophage. After incubating the cells for 30 min at 37°C with 5% CO₂ macrophages monolayers were washed twice with PBS to remove extracellular bacteria. Fresh DMEM with 10% FBS and 1% NEAA were added with gentamicin sulfate (at a concentration of 8µg/ml of media) to the monolayers and incubated for 30 min to kill any remaining extracellular bacteria. Macrophages then were washed twice with fresh DMEM media and incubated for 24 h at 37°C with 5% CO₂.

Detection of VapA by flow cytometry

The J774 cells were counted and plated at 1×10^6 cells/well in a 96-well v-bottom plate. After centrifuging ($300 \times g$ at 4°C for 3 min), the supernatant was removed and the

pellet was resuspended in 60 μ l of blocking solution (10% normal goat serum in PBS) and incubated at 4°C for 20 min. Then 50 μ l of monoclonal antibody against VapA (generously provided by Dr. Shinji Takai, Kitasato University, Japan) were added to each of the wells and incubated for 45 min at 4°C. The plate was then washed 3 times with PBS, incubated for an additional 45 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (IgG; Jackson ImmunoResearch, West Grove, PA, USA). The cells were washed 3 times with PBS and resuspended in FIX buffer (14% formaldehyde in PBS), transferred to a 4ml flow cytometry tube and analyzed on a FACScan BD biosciences flow cytometer (BD biosciences, San Jose, CA, USA). Negative controls included macrophages not infected with bacteria in addition to infected macrophages exposed to secondary antibody without primary VapA antibody.

CHAPTER III

RESULTS

Infection of labeled *R. equi* for phagocytosis determination

To determine whether *R. equi* was phagocytosed by the macrophages, pHrodo™-labeled *R. equi* particles were added in the culture after opsonization and tested on a FACScan flow cytometer. There was significant shift in fluorescence on the histograms of the negative control cells without bacteria and the infected cells (Fig. 1). It was estimated that approximately 30% of macrophages had evidence of intracellular *R. equi* (i.e., phagocytosis). This result affirmed that our technique of infection resulted in phagocytosis, a necessary step for antigen processing by macrophages.

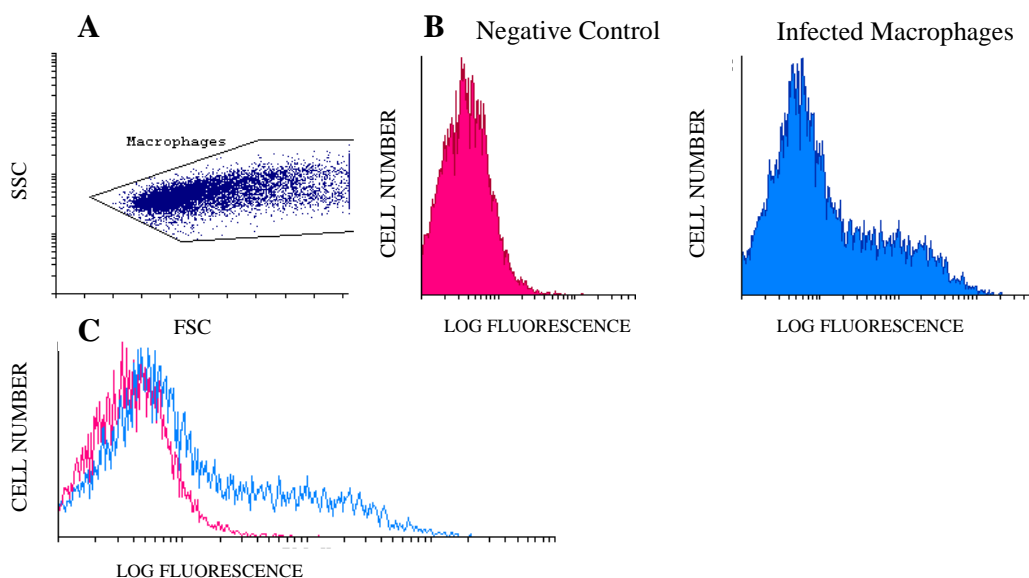


FIG. 1. Infection of murine macrophages with pHrodo™ *R. equi* labeled particles. (A) Dot plot presenting fluorescence of macrophages without labeled *R. equi*. (B) Histograms showing the intracellular fluorescence of phagocytosed *R. equi* (right) compared to negative control macrophages without bacterial infection of *R. equi* (left). (C) Overlay histogram of the previous plots.

VapA expression by *R. equi* infected macrophages

Macrophages infected with a virulent strain (33701+) of *R. equi* bound monoclonal antibody against VapA whereas macrophages infected with an isogenic avirulent strain of *R. equi* (33701-) did not, indicating that macrophages infected with virulent *R. equi* have VapA antigens on their surface (Fig. 2). The experiment between non-opsonized virulent *R. equi* infected macrophages and non-opsonized avirulent *R. equi* infected macrophages were replicated and results were averaged (Table 1); the experiment comparing macrophages infected with either opsonized or non-opsonized *R. equi* (both virulent and avirulent) was conducted only once (Table 1). Using flow cytometry, the average percentage of macrophages that demonstrated binding of the VapA monoclonal antibody to the cell surface was 6.2% for macrophages infected with non-opsonized virulent *R. equi* and 0% for macrophages infected with non-opsonized avirulent *R. equi*. For macrophages infected with opsonized *R. equi*, the percentage of cells with positive results for binding of the VapA monoclonal was 4.5% for the virulent isolate and 0% for the avirulent isolate.

TABLE 1. Expression of VapA by *R. equi* infected murine macrophages

Strain	Percent Positive for VapA expression:		
	Opsonized	Non-opsonized	
		Trial 1	Trial 2
33701+	4.46	2.74	9.56
33701-	0	0	0

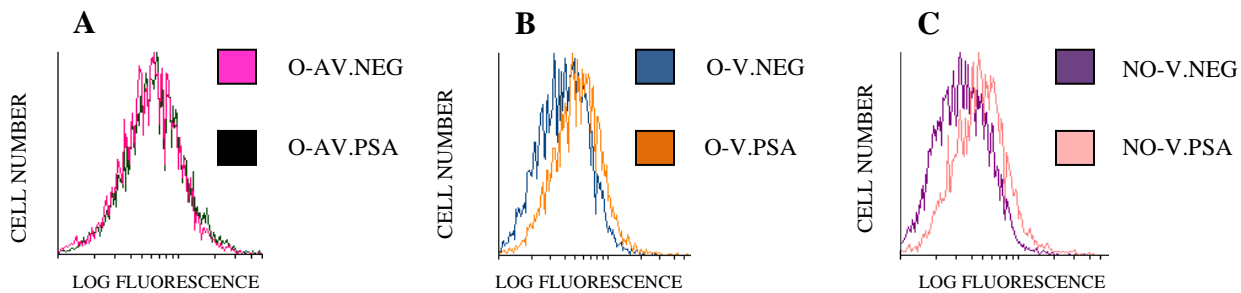


FIG. 2. Expression of VapA by macrophages infected with avirulent (33701-) and virulent (33701+) strains of *R. equi*. (A) Flow cytometry overlay histogram of expression of VapA on macrophage surface infected with opsonized avirulent *R. equi* infected macrophage incubated with secondary FITC conjugated goat anti-mouse IgG antibody (O-AV.NEG), and cells with both primary VapA and secondary antibody incubation (O-AV.PSA). (B) Overlay histogram of opsonized virulent *R. equi* infected macrophages incubated with only secondary FITC antibody (O-V.NEG) and infected cells incubated with both primary VapA and secondary antibody (O-V.PSA). (C) Overlay histogram of non-opsonized virulent *R. equi* infected macrophages incubated with only secondary FITC antibody (NO-V.NEG) and infected cells incubated with both primary VapA and secondary antibody (NO-V.PSA).

CHAPTER IV

CONCLUSIONS

Many experiments have explored the importance of the *vapA* plasmid in the survival and replication of *R. equi* in macrophages, and also the identification of either the VapA protein or the *vapA* gene as a diagnostic test for early *R. equi* detection in foals (7, 8, 11). It has not been reported, however, whether VapA peptides can be detected on the surface of macrophages infected with *R. equi*. In this study, we found evidence that macrophages infected with an isolate of *R. equi* bearing the plasmid encoding the *vapA* gene (i.e., a virulent isolate of *R. equi*), whereas macrophages infected with an isogenic strain of the *R. equi* lacking the virulence plasmid (i.e., *R. equi* which did not express VapA) did not have evidence of VapA antigens on their surface. Knowing that macrophages process and present VapA on their surface when infected with virulent *R. equi* is important for understanding their role in immunity towards *R. equi*. Our findings indicate that macrophages may play a role in antigen presentation to other cells of the immune system following infection with virulent *R. equi*. VapA is known to be highly immunogenic, and evidence exists that antibodies against VapA can modulate the outcome of infection with virulent *R. equi* in mice and foals (1). Thus, infected macrophages might be recognized and destroyed either by antibody-dependent mechanisms or possibly by CTLs. Moreover, it may be important that inactivated or live strains of *R. equi* being evaluated as candidate vaccines can be documented to present VapA on the surface of macrophages or other APCs by which they are recognized.

The percentage of macrophages demonstrating phagocytosis of *R. equi* observed in this report is consistent with but somewhat less than previously reported. Hondalus and Mosser (3) observed 50% of macrophages to have phagocytosed *R. equi*, whereas this experiment observed 30% phagocytosis by macrophages. The reason for the discrepancy between studies is unknown, but may relate to differences in study methods. In the study reported here, phagocytosis was measured in a different murine macrophage cell line (J774.A1) and with a different strain of *R. equi* (33701+) than those used by Hondalus and Mosser. The previous experiment also did not use pHrodo™-labeled *R. equi* particles to flag the presence of intracellular *R. equi*. Collectively, these differences could explain the variation in the percentage of phagocytosis by macrophages reported between studies.

There was no apparent difference in the values observed between macrophages infected with opsonized and non-opsonized *R. equi*; this suggests that the method by which the bacteria are taken up by the macrophage may not determine the extent to which the macrophages present VapA on their surface. The percentage of positive macrophages expressing VapA on their surface was approximately 6%, irrespective of whether the bacteria were opsonized. Although this percentage might initially seem low, it is not entirely surprising for 2 reasons. First, only 30% of infected macrophages phagocytose *R. equi*, such that approximately 21% of macrophages that engulfed *R. equi* had detectable VapA on their surface. Second, we may have underestimated the proportion of macrophages with VapA peptides on their surface. This is because the monoclonal antibody used in this experiment is directed against the VapA protein, but only peptides

are expressed on the surface of macrophages. Peptides are small and linear portions of proteins, many monoclonal antibodies recognize conformational epitopes of proteins. Therefore, our monoclonal antibody might not have bound efficiently or effectively to macrophages with linear peptides that represented only a portion of the VapA epitope(s) it recognizes or because of loss of secondary or tertiary epitope conformation (9).

A limitation of this study is that foals are the species affected by *R. equi* pneumonia, but a murine macrophage cell line was used in this study. Murine cells were used because they have been used previously and demonstrated to behave similarly to equine macrophages infected with *R. equi* (3, 4), and because there is not an equine macrophage cell line available. Repeating the experiments reported here using equine macrophages derived from culture of alveolar macrophages obtained by bronchoalveolar lavage would be valuable. Because adult horses appear to be immune to infection with *R. equi*, whereas (some) foals appear to be susceptible, it would be especially interesting to compare the percentage of macrophages presenting VapA on their surface following infection between macrophages obtained from foals and those obtained from mature horses.

Another limitation of this study is that we did not detect whether the VapA was presented by MHC proteins, and which type of MHC molecules (class I or II) was involved. While one would expect that VapA peptides would be presented in an MHC class I context, this remains to be established by future experiments.

In summary, the finding that VapA expression is found on the surface of macrophages infected with virulent isolates of *R. equi* that express this protein is an important contribution to understanding immunity to *R. equi* infection. This finding suggests new avenues for research, including evaluating detecting VapA on the surface of equine macrophages infected with virulent *R. equi*, comparing macrophages of adult horses to foals with respect to this process, and determining the MHC molecules involved in binding VapA.

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