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**Molecular Pathogenesis of *Mycoplasma bovis*
and *Mycoplasma agalactiae* and its Application
in Diagnosis and Control**

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Molecular pathogenesis of *Mycoplasma bovis* and *Mycoplasma agalactiae* and its application in diagnosis and control

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Continuation of (Related to) Previous BARD Project:☐ Yes ☒ No Number: _____**Keywords** *not* appearing in the title and in order of importance. Avoid abbreviations.**Antigenic and genetic variation, variable surface lipoproteins, molecular typing, epidemiology****Abbreviations** used in the report, in alphabetical order

avg: agalactiae variable gene

MA: *Mycoplasma agalactiae*MB: *Mycoplasma bovis*

Vsp: variable surface lipoproteins

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Abstract

Mycoplasma bovis and *M. agalactiae* are two phylogenetically related mycoplasmas which cause economically significant diseases in their respective bovine or small ruminant hosts. These organisms cause persistent asymptomatic infections that can result in severe outbreaks upon introduction of carrier animals into susceptible herds. Little is known about the mechanisms underlying mycoplasma-host interaction, variation in virulence, or of the factors enabling avoidance of the host immune system. In recent years it has become apparent that the ability of pathogenic microorganisms to rapidly alter surface antigenic structures and to fine tune their antigenicity, a phenomena called antigenic variation, is one of the most effective strategies used to escape immune destruction and to establish chronic infections. Our discovery of a novel genetic system, mediating antigenic variation in *M. bovis* (*vsp*) as well as in *M. agalactiae* (*avg*) served as a starting point for our proposal which included the following objectives: (i) Molecular and functional characterization of the variable surface lipoproteins (Vsp) system of *M. bovis* and comparison with the Vsp-counterpart in *M. agalactiae* (ii) Determination of the role of Vsp proteins in the survival of *M. bovis* when confronted by host defense factors, (iii) Assessment of Vsp-based genetic and antigenic typing of *M. bovis* and *M. agalactiae* for epidemiology of infection and (iv) Improvement of diagnostic tests for *M. bovis* and *M. agalactiae* based on the *vsp*-and *vsp*-analogous systems. We have carried out an extensive molecular characterization of the *vsp* system and unravelled the precise molecular mechanism responsible for the generation of surface antigenic variation in *M. bovis*. Our data clearly demonstrated that the two pathogenic mycoplasma species possess large gene families encoding variable lipoprotein antigens that apparently play an important role in immune evasion and in pathogen-host interaction during infection. Phase variable production of these antigens was found to be mediated by a novel molecular mechanism utilizing double site-specific DNA inversions via an intermediate *vsp* configuration. Studies in model systems indicate that phase variation of VspA is relevant in interaction between *M. bovis* and macrophages or monocytes, a crucial stage in pathogenesis. Using an ELISA test with captured VspA as an antigen, phase variation was shown to occur *in vivo* and under field conditions. Genomic rearrangements in the *avg* gene family of *M. agalactiae* were shown to occur *in vivo* and may well have a role in evasion of host defences and establishment of chronic infection. An epidemiological study indicated that patterns of *vsp*-related antigenic variation diverge rapidly in an *M. bovis* infected herd. Marked divergence was also found with *avg*-based genomic typing of *M. agalactiae* in chronically infected sheep. However, *avg*-genomic fingerprints were found to be relatively homogeneous in different animals during acute stages of an outbreak of Contagious Agalactiae, and differ between unrelated outbreaks. These data support the concept of *vsp*-based genomic typing but indicate the necessity for further refinement of the methodology. The molecular knowledge on these surface antigens and their encoding genes provides the basis for generating specific recombinant tools and serological methods for serodiagnosis and epidemiological purposes. Utilization of these methods in the field may allow differentiating acutely infected herds from chronic herds and disease-free herds. In addition the highly immunogenic nature of these lipoproteins may facilitate the design of protective vaccine against mycoplasma infections.

Significance of major achievements

In this project, we discovered novel genetic systems regulating the expression of genes encoding lipoprotein antigens (Vsps, Avgs) involved in immune evasion and in pathogen-host interaction during *Mycoplasma bovis* (MB) and *M. agalactiae* (MA) infection. These surface components were shown to be: (i) abundant proteins on the mycoplasmas cell surface (ii) highly immunogenic molecules recognized by the host immune system and (iii) subject to high frequency phenotypic switching. During the period of the research, we have conducted an extensive molecular characterization of the *vsp* system and unraveled the precise molecular mechanism responsible for the generation of surface antigenic variation in the mycoplasma (BARD publications #3,#7,#8,#11, see also, ref 1,4,7). Correlation with prior studies on phenotypic variation of MB, and concurrent collaborative studies (see ref: 8) have already demonstrated the importance of this gene family in host-pathogen interactions. Genetic analysis of the *vsp* system revealed a novel mechanism enabling high frequency changes in the expression of the Vsp antigens. We proposed a model for the control of Vsp phase variation that involved double site-specific DNA inversions via an intermediate and unstable *vsp* configuration that occur within the *vsp* locus. Although there are numerous examples of site-specific DNA inversion systems that regulate phase variation of surface antigens, most of them are relatively simple involving a few genes but not a large gene family. In this respect, the *vsp* gene family of *M. bovis* represent unique examples in which site-specific DNA inversion play a major role in regulating the expression of an array of surface lipoproteins. Another significant achievement was the demonstration of the first paradigm in mollicutes, in which chimeric *vsp*-gene fusions, provide an important element of genetic variation and a significant source of antigenic diversification within the mycoplasma population (BARD publication #8). Overall, our research contributes to understanding how these wall-less microorganisms efficiently manipulate their limited genetic material in order to successfully survive within the hostile host environment and to produce chronic infections. Our molecular knowledge on these important surface antigens provides the basis for generating specific recombinant tools useful for serodiagnosis and

epidemiological purposes. These tools will also be important in the rational design of protective immunity against *M. bovis* infections.

As to the pathogenesis of infection of MB, the definition of the host-parasite interactions leading to phase variation of Vsps was approached. The observation that direct contact with bovine blood monocytes was required for ON->OFF phase variation of VspA was novel and supported the initial hypothesis that MB cells shifted to VspA OFF to gain yet undescribed advantage when interacting with monocytes. In contrast, interaction with epithelial cells such as MDBK, or ciliated respiratory epithelial cells of tracheal explants led to only minor or undetectable ON->OFF phase variation of VspA in MB. This information is crucial to narrowing the focus of future research on Vsp phase variation to stages of pathogenesis where the mycoplasma has to interact with macrophages or monocytes.

The rapid phase variation of VspA also is an indication that pre-existing signals on the membrane of the monocyte are probably involved. The use of tracheal explants as models for respiratory epithelium and other *in vitro* model systems have important limitations, which must be taken into account when attempting to extrapolate to *in vivo* host-parasite interactions.

We made significant progress in delineation of an analogous gene system in MA, first described in this project (BARD publications #1,#6). Comparison of the two systems in two pathogenic mycoplasmas has important scientific value and the first step toward understanding pathogenesis of MA which has never been explored. Our field studies with MA, which clearly demonstrated genomic rearrangements in the *avg* locus during chronic infection, are also innovative. Ongoing studies in our laboratories are attempting to identify the molecular characteristics of MA and the cognate host factors that determine the course of infection. A logical potential development will be similar studies of chronic infection in MB infected animals, in which the relationship between genomic rearrangements and phenotypic variation are much more clearly understood.

An additional project goal was the development of diagnostic tools for MB and MA based on molecular characterization of pathogenesis of infection. Serological

observations, performed by ELISA using captured VspA as antigen, indicated that phase variation occurs *in vivo* and under field conditions, and is thus an important element in the interaction of MB with the host. Serological methods of detection of infection are still preliminary. If these methods can be further refined, it may be possible: (i) to differentiate acutely infected herds from chronic herds and disease-free herds; (ii). to differentiate vaccinal antibody responses from infection-associated responses. Due to the time frame of the project it was not possible to exploit recent molecular information regarding non-VspA members of the *vsp*-gene family in MB, or the *avg*-gene family in MA for diagnostic tests which could be validated with clinical samples.

Potential for applications

Immediate: Although focus in the VMRI research group is shifted away from Vsps as diagnostic tools or vaccines, contemporary collaborative and independent studies in Europe regard this gene-family as the key factor in MB pathogenesis and diagnosis. Project-related molecular studies are already being applied in this direction. Evaluation of *avg*-related serological screening methods for MA in Israel is a high priority goal for application.

Medium term: Improved serological methods will be useful in regulation of animal movement, among herds in regions or between countries differing in MB or MA disease status. In the USA this is particularly important for dairy farms, where the trend is to replace production cows with heifers raised by specialized farms. In Israel this would help prevent introduction of MB with import of animals from highly infected regions, as well as furthering control of MA infection in Israel from indigenous or imported sources.

Long term: New vaccines will make control of MB and MA disease possible. Eventually, eradication of the infection can be contemplated, particularly when vaccination is combined with a refined serological detection test that can differentiate vaccinal responses from infection responses.

Estimated economic benefits and potential economic benefit

In the USA, economic losses due to MB mastitis mount to \$108 million/year, MB calf pneumonia losses reach \$4.4 million/year, and feedlot pneumonia losses are \$27.5 million/year. These estimates were derived from incidence of infection estimates obtained in 4 separate field and diagnostic surveys performed by VMRI in the Midwestern USA, coordinated with data obtained from surveys performed by others in other geographic sets. When combined, resolving the problem of MB in the US could result in reductions of losses of nearly \$140 million/year. Losses due to MA outbreaks in Israel (8-10 each year) were estimated in two cases at \$64,000 and \$222,000 (BARD Publication #4), but lesser dollar damage may be devastating for small growers.

In general, widespread use of diagnostic tests for MA and MB, as well as continuing education provided to veterinarians has increased awareness of diseases caused by these organisms. This has resulted in earlier diagnoses, shifts in therapy and management of the diseases, and in many cases, significant reductions in mortality and treatment costs. Taken together, an aggressive application of the above strategy by VMRI has resulted to date in an estimated approximate 20% reduction of the cost to producers caused by MB infections. Recognition of the importance of diseases caused by MA and MB has caused a resurgence in industry interest in vaccine development. VMRI is now funded by industry for a multi-year effort to develop a vaccine against MB and the Israeli group is initiating small-scale research on an MA vaccine with local funding.. Availability of a vaccine together with improvements in serologic detection of infection, should also impact on the cost of MA and MB diseases.

Details of collaboration

The Israeli groups at HUHMS and KVI are completely collaborative on all levels of interaction. At the early stages of the project, collaboration with VMRI was well established and there was an active interchange of information and reagents, and coordination of project strategy. For objective reasons, there were fewer interactions during the last year of the project and the focus of VMRI research shifted somewhat

from the major goals in this project. One result was that project strategy was not fully coordinated and research achievements, on both sides, were not fully exploited for advancement of project objectives.

Background

Mycoplasmal infections are prevalent in man and animals, characterized by chronic colonization of mucosal surfaces and generally mild clinical disease forms (9). However, some species of mycoplasmas can cause serious disease or manifest themselves in diverse clinical forms by virtue of their ability to produce a systemic infection. *Mycoplasma bovis* (MB) and *M. agalactiae* (MA) are two phylogenetically related mycoplasmas which cause economically significant diseases in their respective bovine or small ruminant hosts (2,3,5). In addition to many varied clinical manifestations of MB or MA infection, these organisms can cause persistent asymptomatic infections that may result in severe outbreaks upon introduction of carrier animals into susceptible herds. Little is known about the mechanisms underlying these early events in mycoplasma-host interaction, variation in virulence, or of the factors enabling avoidance of the natural immune host-mediated defense systems. However, it has become apparent in recent years that the ability of pathogenic microorganisms, including the mycoplasmas, to rapidly alter surface antigenic structures and to finetune their antigenicity (**antigenic variation**) is one of the most effective strategies used to escape immune destruction and to establish chronic infections (6,10,11).

The starting point for this proposal was our discovery of a novel and complex genetic system encoding a family of surface lipoproteins (**Vsps**) undergoing high frequency phenotypic switching in MB (4) and preliminary evidence for the presence of an analogous system in MA. The major working hypothesis of this project was that the Vsps of MB have an important role enabling the organism to efficiently evade host defense mechanisms. We addressed this issue by molecular and functional characterization of the *vsp* gene system of MB and determination of the role of the Vsp proteins in the survival of MB when confronted by host defense factors, using

model systems. Our second hypothesis was that MA employs a similar strategy of adaptation to the small ruminant host environment, which we proposed to study *in vivo* in naturally infected animals. The molecular analysis of the *vsp*-analogous gene family in MA (designated agalactiae-variable-gene, *avg*) and the comparison to the MB *vsp*-gene family are essential for understanding the survival of the two mycoplasma species within their natural host as well as their pathogenic capability. Our third hypothesis was that molecular tools based on the *vsp/avg* genes and their products would be valuable tools for improving the diagnosis of MB and MA infection. We proposed to assess this on two levels: (i) the utilization of *vsp/avg* genetic and antigenic variation as markers for study of epidemiology of infection; (ii) improvement of diagnostic tests for MB and MA by exploiting molecular knowledge of these major immunogenic surface antigens.

Methodologies and Materials

Previous work at Hebrew-University Hadassah Medical School (HUHMS) has shown the presence of the Vsps undergoing variation in expression and size (1,4,7). Two valuable tools prepared in this study allowed efficient identification and molecule analysis of the MB *vsp* gene system. The first was a clonal lineage representing several sequential isolates derived from a single strain and exhibiting oscillating phase transitions of VspA, i.e., ON $\rightarrow$ OFF $\rightarrow$ ON (1,4). The second tool was a set of synthetic oligonucleotides representing sequences complementary to distinct regions of the *vsp* coding regions. By using these oligos as probes in Southern blot hybridization against restricted genomic DNAs of Vsp phase variants we could readily monitor genomic fragments undergoing high frequency rearrangement during Vsp phase transition. To clone the *vsp* system, MAAb 1E5 (kindly provided by Dr. K. Sachse, Jena, Germany), previously shown to recognize a common epitope present on three distinct Vsps (1,4,8), was used to screen MB expression genomic library constructed in the bacteriophage λ GEM-12 (Promega) from a clonal isolate with the expression phenotype VspA/VspB (63-kDa VspA and 46-kDa VspB). Other

molecular methods utilized in this project to genetically analyze the *vsp* gene complex, as well as the *avg* gene system, were previously described (BARD publications #1,#3,#6,#7,#8). Methodology for isolation of MB and MA from clinical samples, identification and growth in culture are described in BARD publications #1,#2,#3, and #6. Clinical samples were obtained from diagnostic field laboratories and from project-initiated field studies in the USA (MB) at the Veterinary Medical Research Institute (VMRI) and in Israel (MA and MB) at Kimron Veterinary Institute (KVI).

Sequence data of VspA, made available by HUHMS (4), was used to generate rabbit antisera to 4 separate repetitive regions of VspA (R_A1 - R_A4) and to a 13 amino acid sequence spanning the R_A2 and R_A3 repeats (region 13AA). Clonal variants of MB strain PG45 expressing VspA (CV6) or not expressing VspA (CV7) were received by the VMRI from HUHMS. An RNase protection assay was developed to detect a transcript of a 309 bp spanning the VspA mRNA, using a 360 nt MB rRNA as internal control (unpublished results)

Results and Discussion

The vsp genomic locus of M. bovis

Efforts during the last three years have led to the identification and functional characterization of the *vsp* system mediating antigenic variation in MB (BARD publications #3, #7, #8, #11). This genetic system plays apparently an important role in the complex interaction of the bacterial pathogen with its host, in determining the chronic nature of the mycoplasmal infection and in particular, enables the mycoplasma efficient avoidance of host defense mechanism.

The *vsp* genomic locus of about 23 kb contains a large family of 13 single-copy *vsp* genes each of which exists as independent transcriptional unit encoding a putative surface lipoprotein (Fig. 1 of BARD publication #3). Several striking aspects of Vsp structural similarity sequence divergence and variability were revealed (Figs. 2 and 3 of publication #3). Each *vsp* gene is preceded by a highly conserved 5' non-coding sequence that can be divided into two internal cassettes. The first (cassette #1), a 71 bp region upstream of the ATG initiation codon, exhibits 99% homology among all

vsp genes. The second region (cassette #2), of about 150 bp, is more divergent. The Vsp N-terminal region of 25 amino acids (aa) is also a highly homologous domain showing 99% aa identity among Vsps and representing a typical prokaryotic lipoprotein signal peptide. These lipoproteins were shown to be the most abundant amphiphilic proteins in MB. In contrast to the highly conserved nature of the *vsp*-5' upstream region and of the N-terminal domain, there is a considerable sequence divergence among mature Vsp molecules. Examination of the deduced Vsp aa sequences revealed an unusual structural motif. Most of the Vsp molecules are composed of reiterated coding sequences extending from the N-terminus to the C-terminus of the protein chain, thus creating a periodic polypeptide structure. The substantial sequence divergence of the mature protein in the Vsp family is generated by 18 distinct reiterated units of different amino acid sequences and length, which are distributed within the Vsp molecules. The majority of these repeated sequences are arranged in the form of tandem domains comprising up to 80% of the entire Vsp molecules (see BARD publication #3). Recently, these repetitive domains were found to possess immunogenic epitopes as well as adhesive structures (8). These findings are consistent with the highly immunogenic nature observed for the Vsps and suggest a possible function for these molecules as adherence-mediating regions in pathogenesis.

The molecular genetic basis of Vsp phase variation

One of the striking aspects of the *vsp* system was the link between changes in the antigenic repertoire and rearrangement events occurring at high frequency within the *vsp* locus (4). Noteworthy, although the *vsp* locus contains at least 13 distinct *vsp* genes, clonal isolates expressing the VspA product were found to possess only one active *vsp* gene: the *vspA* gene ((BARD publications #3 and #7). Since the other *vsp* genes were apparently transcriptionally silent in these isolates, we were able to monitor and characterize the precise nature of the rearrangement events associated with high-frequency phenotypic switching of a single Vsp, namely the VspA protein. Molecular analysis of the *vsp* locus from the three VspA phase variants has shown

that the phase variable *vspA* gene as well as all other *vsp* genes possess complete and uninterrupted ORFs regardless of their expression state. Based on experimental evidence the probable conclusion is that silent *vsp* genes are inactive due to the lack of a promoter (BARD publication #9). In this paper we provided experimental evidence demonstrating that phase-variable expression of the *vspA* gene is regulated by site-specific DNA inversions. As a result, an interchange of active (*vspA*) and silent (*vspO*) *vsp* genes occurs, leading to the fusion of the silent *vspO* gene to the A₂ cassette bearing the promoter region, and subsequently to its expression (Figs. 2, 5 and 7 of BARD publication #7)

Further studies of the site-specific DNA inversion mechanism regulating VspA phase variation in MB emerged when the *vsp* gene configurations in the three VspA phase variants were compared. The *vsp* configuration present in the VspA-OFF variant could not be explained by a single site-specific DNA inversion event occurring during VspA ON/OFF switching (ON→OFF→ON). Our model for the molecular mechanism for genomic rearrangement for the *vspA* gene, and supporting experimental evidence, are presented in BARD publication #7. The data clearly indicate that the control of Vsp phase variation in MB necessitates two distinct and successive site-specific DNA inversions. To our knowledge, the *vsp* system represents the first example among the known bacterial site-specific DNA inversion systems in which phase variation of surface lipoproteins is mediated by double site-specific DNA inversion occurring at a high frequency.

Significantly, VspA phase variation was shown to be phenotypically and genotypically reversible, at least in the case of the three clonal isolates tested (4). In other words, the double site-specific DNA inversion model works apparently in identical mode in both directions. Variation in the antigenic repertoire is a combination of the frequency of the genetic switch and of the selective pressure of the environment for a particular surface protein, which may be very high if those proteins are recognized by the host immune system. The VspA protein has been shown to be an abundant surface protein and highly immunogenic (1,7). Further studies are needed to determine *in vivo* whether certain site-specific DNA inversion patterns are preferable

in the host as a result of the selective pressure provided by immune system.

The vsp-related genes of M. agalactiae and the relationship to the gene family in M. bovis

Using the *vspA* gene as a probe we identified in the chromosome of MA multiple *vsp*-related genomic fragments (BARD publication #1). Using the methods described in BARD publications #3 and #6, four *vsp*-related genes have so far been identified and designated as *avg* genes (*avgA-D*). These *avg* genes display a significant homology at their promoter region, as well as in their N-terminal-encoding end, to the corresponding regions of the *vsp* gene family of MB. (Figs. 1 and 2 of BARD publication #6). However, sequence analysis of the four-*avg* genes has shown the lack of *vsp*-like repetitive domains on one hand, and also the complete absence of any *avg* reiterated coding sequences on the other hand. Since a significant portion of the *vsp* genes are composed of repetitive coding sequences, enabling the Vsp molecules to undergo high-frequency size variation (ref. 1,4 and BARD publication #3), the lack of such units within four members of the *avg* gene family represents a fundamental difference between the two gene families, and suggests that at least four Avg proteins are not subject to size variation. Molecular characterization of the rest of the *avg* locus is currently being carried out.

Examination of the *avg* genomic fingerprints of MA clinical isolates *in vivo* indicate that the *avg* locus is a dynamic region undergoing *in vivo* variation (BARD publication #1, #2, #6). By using *avg* specific oligonucleotide probes we could detect two genomic fragments carrying the four-*avg* genes undergoing rearrangement. Preliminary sequence analysis of the rearranged fragments in the sequential isolates revealed that a recombination event leads to repositioning of the *avgB* gene in its opposite orientation. Rearrangement events within the *avg* locus resemble a key feature of the Vsp family of MB (as described above)

The role of Vsp proteins in the survival of M. bovis

Work done in the USA (VMRI) was directed towards understanding the role of Vsp proteins in the survival of MB when confronted by host defense factors, using model systems. Preliminary data have shown that antigenic variation in Vsp expression was operating *in vivo* in field infected cattle with abscessing lesions (unpublished results). This led to the concept, presented in the proposal, that contact of MB with host cells involved in defense against infection had profound effects on Vsp expression. Several *in vitro* models were explored to confirm and extend these observations. Data presented in Data Appendix #1 describe the development and validation of the antibody reagents used in these studies, and their application to characterize clonal variants of MB.

Phase variation studies were conducted using suspensions of various mammalian cells and variants CV6 (>95% VspA expression=VspA-ON), and CV7 (0% VspA expression=VspA-OFF) of MB, as described in Data Appendix #1. Bovine blood monocytes caused significant ON->OFF phase variation of VspA, with drop to 22% VspA-ON at 1 hr of reaction. VspA expression then progressively returned, reaching initial values by 6 hrs of reaction (Data Appendix #1, Fig. 1). Other bovine cell types tested did not have this effect on VspA expression, with the exception of MDBK cells that exhibited a small but significant ON->OFF phase variation effect (Appendix#1, Table. 1). The observation that direct contact with bovine blood monocytes was required for ON->OFF phase variation of VspA was novel and supported the initial hypothesis that MB cells shifted to VspA OFF to gain yet undescribed advantage when interacting with monocytes. Further data are presented in Data Appendix #1. In efforts to further define the phase variation interaction, transmission electron microscopy of cell pellets was performed. Preliminary data relating to cellular responses of the mammalian cells in response to phase variation in the above described model systems are also presented in Appendix #1.

Molecular typing for persistent infection/acute infection

One of the most important aspects of the project at KVI and HUHMS was the genetic analysis of MA in chronically infected asymptomatic sheep. We started from scratch—at the onset there was virtually no information available for either MA or MB. Elucidation of the *avg*-gene family in MA, described in detail above and in BARD publication #6, was carried out mainly with samples from this case study. The genetic tools, which we now have in hand, are being applied to address the underlying question: what is the mechanism by which MA infection may, in some cases, result in a persistent chronic infection.

As described in BARD publication #6, a longitudinal case study was carried out in a dairy sheep flock located in a village in northern Israel in which samples were collected at 2-4 week intervals over a period of ten months. Persistent infection was studied in sequential MA isolates from three animals. Isolation of MA was made from the prototype animal ,#627, at each sampling time (BARD publication #6 and Data Appendix #2). Data from the other two animals present molecular evidence for reinfection of animals from asymptomatically infected flockmates (see, Data Appendix #2). In addition, there is evidence for establishment of an “*avg*-subtype” which appears to be preferentially present in persistent infection. It remains to be established if this particular “*avg*-subtype” has properties which allow this MA strain to escape normal host defenses and to establish chronic asymptomatic infection. This will be investigated in the continuation of the project, using archived isolates and serum samples from all the animals.

A second case study compared the genomic fingerprints in sequential MA isolates in an acute outbreak of CA, followed at weekly intervals over a period of about two months. Preliminary data are presented in Appendix #2. Notably, the *avg* genomic fingerprints were highly homogeneous among the different animals and in successive isolates from the same animal. This was in marked contrast to the heterogeneity observed among animals and with time in the chronically infected flock.

Diagnostic tools and epidemiology

Diagnosis of MB in cattle is a major and on-going concern at VMRI and interaction with the industry is maintained by the high level of service activity and reflected partly in the list of relevant presentations to various professional organizations (see publications list). Within and beyond the scope of the BARD project, VMRI has put in place a battery of diagnostic tests for use in MB infections in the US. In previous project reports, results were reported of use of a spot immunoblotting test for detection of MB in milk, using a pool of MAbs including MYB87 (reactive with VspA and VspC). It was found that spot-immuno-binding could not be used as the sole detection method. Immunohistochemistry using MB-specific Mabs has established itself as a validated diagnostic method for respiratory infections where mortality gives access to lung tissue lesions. Culture continues to be the method of choice for cases with arthritis where joint fluid can be accessed, and for dairy herd screening via bulk milk tank samplings.

The nested PCR test was developed for detection of MB in milk (BARD publication #10). Enhancement of a previously reported PCR test recognizing MB-specific non-*vsp* related gene together with development of an innovative rapid procedure for processing milk samples resulted in a significant increase in sensitivity compared to culture. The described method for processing of milk samples, which eliminates inhibitory substances present in these clinical samples, has potential for application of PCR tests based on conserved regions of the *vsp* gene family, now under development.

Development of serological tests to evaluate exposure of dairy and beef herds to MB is a priority for the US. VMRI has approached this problem by devising antigen capture ELISA serology with three different MAbs (Data Appendix #3), and comparing the results with those obtained with a whole antigen ELISA. Eight herds with known clinical history, totaling 1968 animals, were tested. From the data it may be concluded that antibody responses in this test to VspA and VspC were not useful as diagnostic indicators of acute or chronic infection..

At VMRI, an epidemiological study was carried out using a combination of three

different typing methods of evaluation (BARD publication #9). Genetic characterization of MB isolates was carried out by arbitrary-primed PCR; antigenic phenotypes were determined using specific MAbs (Vsp-related and non-related); and the putative pathogenicity factor, cytotoxicity, was measured. MB isolates from three herds differing in epidemiological and clinical parameters were tested. Two closed cattle herds were each infected from a single source, as shown by uniform PCR typing patterns. Isolates from these herds were also uniform phenotypically initially, but diverged with time, as measured by surface antigen presentation and cytotoxicity. All three methods indicated heterogeneity among isolates in a herd in which animals were introduced from multiple sources that differed in the MB strain present. Patterns of *vsp*-related antigenic variation, as measured by MAb MYB87, diverged rapidly within the closed herds, irrespective of the PCR type. The phenotypes detected were compatible with the molecular mechanism for turning OFF and ON of these phase-variant surface proteins, as elucidated in this project (BARD publication #7).

In Israel, MB infection remains only a sporadic problem, mainly associated with calves imported from Europe for slaughter. Preliminary tests evaluating the use of immunohistochemistry for diagnosis of MB in lung samples were carried out within the framework of the project, with exchange of paraffin-fixed samples and reagents between KVI and VMRI. The feasibility of the test was demonstrated but there was no follow-up due to the lack of clinical samples.

Diagnosis of MA infection in Israel continues to be carried out by classical methods, with the main impact of the project reflected in increased efforts to evaluate the full extent and epidemiology of infection (BARD publications #2,#4).

Major efforts were devoted to evaluation of the *avg*-gene family in MA as a tool for epidemiology. Genomic fingerprints of the *avg* locus were determined by the use of one or several probes (BARD publications #1, #2, #6), examining *Hind* III restricted DNA from MA isolates. Samples tested were from three different sources: (i) Isolation from chronically infected animals, at the same sampling time; (ii) Isolates from acute Contagious Agalactia, as described in the above described case study; (iii)

Multiple isolates from clinical outbreaks, received in the course of case submissions to diagnostic labs.

Preliminary and partial results have been presented (BARD publications #1,#2,#6) and are included also in Data Appendix #3. On the whole, MA isolates from different animals at the acute stage of infection showed a high degree of homogeneity. On the basis of these patterns we were able to describe an outbreak-representative pattern. This was based on the predominance of MA isolates with the same “avg-subtype”. In several cases we were able to detect marked differences in the representative patterns between outbreaks which correlated with independent epidemiological information. However, due to the complexity of the genomic patterns using the tools that were available at that stage of the project, we are not yet able to evaluate the potential of this approach to molecular typing and the possible application for epidemiological studies. We have carried out only preliminary studies of antigenic variation in MA, using convalescent and acute sera from naturally infected animals. Elucidation of the sequences of the *avg* genes described above has been only recently completed. The next step is to express these genes *in vitro* and produce cognate MAbs and monospecific antibodies, which will enable correlative studies of genetic and antigenic variation.

Scientific importance and implications for agriculture

In recent years there is an increasing awareness of the importance of antigenic variation as a strategy for evading host immune system in many bacterial pathogens. The discovery and the genetic characterization of the *vsp* system, which mediates antigenic variation of the major immunodominant surface lipoproteins in MB, and the elucidation of the molecular genetic basis for phase variation of this system are of no doubt important achievements. The complexity and sophistication of this important mechanism for evasion of host defence mechanisms are elegantly and comprehensively described in a series of project-related scientific papers which have received very positive peer-recognition and feedback. The *vsp* system is recognized as a prototype and touchstone for comparison of similar systems in other important animal and

human microbial pathogens. An important example is the *avg* gene family in MA, described for the first time in this project. The economic significance of MB and MA is as important at this time as when the proposal was submitted and the project initiated. Project-related diagnostic activity and field studies, together with independent studies in the USA and Europe, provide current documentation of the continuing, and growing, need for improved control of these diseases. The results of this project relate to the molecular pathogenesis of these organisms and also provide the molecular basis for improved diagnostic tools or immune prophylaxis. All the information will be readily accessible to the scientific community, and thereby will facilitate rational policy decisions regarding control strategies

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Data Appendix #1

The role of Vsp proteins in the survival of MB when confronted by host defense factors, using model systems and experimentally infected animals (Objective #2 in the BARD research proposal)

Preliminary data was available to indicate that antigenic variation in Vsp expression was operating in-vivo in field infected cattle with abscessing lesions. Abscessing strains of MB can produce systemic infections in cattle with production of abscesses in lung tissue, joint capsule wall, and walls of tendon synovial sheaths. In these abscesses, MB is detected by immunohistochemistry and is found in both the center and periphery of the lesion. When MYB87, a monoclonal antibody recognizing VspA and VspC was used as detecting reagent, it was found that MB cells located in the center of abscesses expressed Vsps, while MB cells located in the periphery of the abscesses did not. It was also noted that while neutrophils were the predominant cell type in the center of abscesses, mononuclear cells, and in particular, macrophages and histiocytes predominated on the periphery. This led to the concept that contact of MB with host cells involved in defense against infection had profound effects on Vsp expression. The following observations are the subject of manuscripts now in preparation.

Several in-vitro models were explored to confirm and extend these observations. To use these models, antibody reagents had to be validated or developed, and clonal variants of MB received from IUHMS. It was found that the monoclonal antibody MYB87 reacted not only with VspA and VspC, but also with a smaller uncharacterized Vsp. This latter reactivity was prominent with several US strains of MB, but minimally present in strain PG45, the strain used by HUHMS for the characterization of Vsp genes. In addition, since the sequence of VspA was made available by HUHMS, rabbit antisera to 4 separate repetitive regions of VspA (regions RA1 through RA4) and to a 13 amino acid sequence spanning RA2 and RA3 (region 13AA) were prepared. Clonal variants of MB strain PG45 expressing VspA (CV6) or not expressing VspA (CV7) were received from HUHMS. Variants CV6 and CV7 were shown to express VspA, VspB, and the small Vsp, when tested with rabbit anti-MB antisera or rabbit antisera to VspA peptides. It should be noted that antisera to RA3 reacted with native and denatured forms of VspA and VspB since this repeat is common to both Vsps. In contrast, rabbit antisera to RA1, RA2, and 13AA reacted specifically and only to native VspA. In separate work, a RNase protection assay was developed to detect transcription of a 309-nucleotide span of VspA mRNA, using a 360 nt MB rRNA as internal control.

Phase variation studies were conducted using suspensions of various mammalian cells and variants CV6 and CV7 of MB. Bovine blood monocytes were obtained from healthy donor cattle by collection of buffy coat layers followed by flash hypotonic lysis of erythrocytes and Percoll gradient centrifugation. The preparations contained 60-90% monocytes with residual lymphocytes and dendritic cells. Blood neutrophils were obtained from erythrocyte pellets followed by flash hypotonic lysis of erythrocytes. These preparations were >95% neutrophils with residual lymphocytes. Blood lymphocytes were obtained by Ficoll-Hypaque gradient centrifugation with >95% purity and residual neutrophils. Other cell types used were routinely cultured, including diploid bovine lung fibroblasts, an established SV40-transformed bovine monocyte cell line (BOMAC), and bovine kidney epithelial cells (MDBK). Variants CV6 and CV7 were grown in broth from frozen stocks and evaluated for VspA expression by contrasting colony lifts reacted with 13AA antiserum from lifts reacted with anti-MB antiserum. Twenty-hour log phase broth cultures of CV6 generated a population of colonies with >95% VspA expression (VspA-ON), while corresponding CV7 broth cultures generated a population of colonies with 0% VspA expression (VspA-OFF). Reactions involving MB variants and mammalian cells were set up in replicates with 100 mycoplasmas per cell and 5×10^5 cells/ml, and tumbled at 37C. Samples were retrieved at specified times between 0 and 6 hrs. At each sampling, mycoplasmas unreacted with cells, reacted but unattached to cells, or reacted and attached to cells (by differential cell lysis and detachment using sonication) were evaluated.

Bovine blood monocytes caused significant ON->OFF phase variation of VspA, with drop to 22% VspA-ON at 1 hr of reaction. VspA expression then progressively returned, reaching initial values by 6 hrs of reaction (Fig 1).

MN/B136

Fig 1. Phase variation in variant CV6 (VspA ON) reacted with bovine blood monocytes. Mycoplasmas attached to monocytes underwent rapid ON->OFF phase variation, detectable at 1 and 2 hours, while unattached mycoplasmas remained in ON phase.

Other bovine cell types tested did not have this effect on VspA expression, with the exception of MDBK cells that exhibited a small but significant ON->OFF phase variation effect (Table 1). It was important to note that bovine lymphocytes did not induce ON->OFF phase variation of VspA, since these cells were present in preparations of bovine blood monocytes. Of note also was the observation that only cell-adherent fractions of MB exhibited ON->OFF variation, while non-adherent MB were not altered. This was confirmed by RNase protection assays of non-adherent fractions of MB when compared to MB not exposed to mammalian cells. There was no decrease in VspA mRNA production of non-adherent MB cells. Unfortunately, the adherent MB fraction could not be tested by RNase protection assays due to the small amount of mRNA recoverable (3 MB organisms adhered/cell on average under the test conditions). Cultured mammalian cells also contributed mRNA that reacted nonspecifically with the protecting VspA mRNA fragment assayed, complicating interpretation of the data. In efforts to further define the phase variation interaction, transmission electron microscopy of cell pellets was performed. Mycoplasma organisms were seen interacting with bovine blood monocytes and lymphocytes in equal proportions. In these preparations, a few dendritic cells were observed, and these interacted with MB organisms that were elongated or cigar-shaped. The ratio of MB organisms to dendritic cells was similar to that seen with monocytes or lymphocytes, but only on dendritic cells were MB organisms changed in shape. When the CV7 (not expressing VspA) variant was used, all samples containing MB remained in OFF phase mode, regardless of the cell used or time of reaction tested. It was interesting to note that as with the CV6 variant (expressing VspA), 3 MB organisms/cell would attach on average.

Table 1. Percent change in ON->OFF phase variation in VspA expression of *M. bovis* after direct contact with mammalian cells for 1 hour.

Cells tested	Origin	Cell type	% Change (C.L.*)
Blood monocytes	Bovine	Macrophage	71 (78; 63)
BOMAC	NADC ¹	Macrophage, transformed	0
MDBK	ATCC ²	Epithelial	15 (31; 5)
Bovine lung	R Rosenbusch	Fibroblastic	4 (20; 0)
Blood Neutrophils	Bovine	Neutrophil	3 (17; 0)
Blood lymphocytes	Bovine	Lymphocyte	0

* (C.L.) Confidence limits for % change calculated at $p < 0.05$ and expressed as (High; Low) limits. Significant % change was detected if Low limit > 0 .

¹ NADC Dr. J. Stabel, National Animal Disease Center, USDA, Ames, IA.

² ATCC American Type Culture Collection, Rockville, MD

Some initial attempts were made to explore the phase variation model from the perspective of the mammalian cell. Blood monocyte preparations with or without MB were tested for release of TNF-alpha into supernate. The lymphokine was tested with a biological test for cytotoxicity to target L929 cells, and activity compared to that of the recombinant lymphokine. Release of TNF-alpha into supernates occurred late (1400 pg/ml at 4 to 6 hrs) and in low amounts from blood monocytes reacted with CV6 variant of MB. Supernates from monocytes left unreacted with MB, or from MB without monocytes did not contain detectable TNF-alpha. In other trials, cultured mouse macrophages were tested for ability to induce ON->OFF phase variation in the CV6 variant of MB. The established mouse macrophage cell lines P388.D1, J744A.1, and IC-21 were tested for this activity and only P388.D1 cells were found to elicit it. These cells reduced VspA expression to 75% of original levels, a much smaller effect than that seen with bovine blood monocytes.

As part of Objective 2, the effect of complement and anti-VspA antibody, as well as the interaction of MB with respiratory epithelial cells was to be explored. Early in the course of the project, another research group published evidence that antibody and complement could interact in-vitro with a population of MB expressing VspA, resulting in the appearance of MB not expressing VspA after 6 days of culture. This led to our early decision not to pursue further the role of antibody and complement on VspA phase variation. The role of bovine respiratory epithelial cells on MB Vsp phase variation was tested using cultured tracheal explants and immunohistochemistry. In initial tests, MB organisms were seen to interact with ciliary processes of ciliated respiratory epithelial cells at 1 and 2 hours after reaction. These MB organisms were continuing to express VspA (detected with monoclonal antibody MYB87). Later in the interaction, it appeared that MB organisms were seen in intercellular spaces between ciliated respiratory epithelial cells, or in linear arrays radiating towards blood vessels in the submucosa. These antigen-associated signals were only detected with monoclonal antibody MYB163, and not with MYB87. When (F(ab)₂ fragments of rabbit anti 13AA were used to replace MYB87 and (F(ab)₂ fragments of rabbit anti-MB were used to replace MYB163, we could only confirm that MB attachment to ciliated processes of respiratory epithelial cells occurred while VspA is expressed in variant CV6 of MB. Translocation across the respiratory epithelium was only seen in areas with rounded/altered epithelial cells, and MB organisms in these sites still expressed VspA. This is an area of continuing studies for VMRI.

Data Appendix #2:

The role of the avg-gene family in persistent /acute infection

One of the most important aspects of the project at KVI and HUHMS was the genetic analysis of MA in chronically infected, asymptomatic sheep. As described in BARD publication #6, a longitudinal case study was carried out in a dairy sheep flock located in Manshia Zabda, a village in northern Israel. MA was isolated from severe clinical Contagious Agalactia in the flock in 1996 and early 1997, but only sporadic clinical manifestations were observed during the course of this study, which was initiated in July 1997. MA isolates from milk and serum were collected from eight individual asymptomatic animals at 2-4 week intervals over a period of ten months. MA was identified by standard methods and archived at about the 4-5 passage level. Isolates were not filtered cloned but were homogeneous by IMF. DNA for Southern Blot hybridization and protein for Western Blot analysis were prepared by standard laboratory methods. The genetic tools used for analysis of the MA isolates were the oligo probes sig-1, A-1, and B-1 and a 0.6 kB probe encompassing part of genes *avg A* and *avg B*. The position of these probes is indicated on the schematic diagram of the *avg* locus (Fig. 1 in BARD publication #6). For comparison of hybridization patterns among the Hind III-restricted DNA samples from different animals, or comparison of results with different probes, data was summarized in tabular form. Size estimates are relative to standard DNA size markers and compared to the pattern for MA type strain PG-2 that was included as a standard in all tests.

Table 1 shows the marked variation in the band patterns among the test animals at the onset of the trial. All the strains differ markedly in hybridization from the pattern of the MA reference strain PG-2, but to a lesser degree they differ among themselves. Seven out of the eight strains tested differ in the hybridization pattern, with only isolates from #613 and #617 showing identical patterns.

Table 1: Band patterns revealed by the sig-1 probe hybridization in DNA from MA isolated at sampling time 4 (start of longitudinal study).

kB	PG-2	608	611	613	617	618	619**	626	627
6.0		+		+	+	+	(+)	+	+
5.3	+		++				++	+	
4	+	+	++	+	+	+	+	+	+
3	+	+	+	+	+	+	+		+
2.5	++							+	
2.2		+	+	+	+	+	+		+
2.0		+		+	+		+	+	
1.8		++							++
1.5						+			
1.3	+	+	+	+	+	+	+	+	+
1.1			+	+	+	+	+	+	
1.0	+								
0.6			+				+		

* indicates doublet band. (+) or (-) indicates minor shift in band size relative to other samples. All size estimates are approximate, based on DNA size marker.

** indicates multiple bands, some shared with other isolates and some unique.

The sig-1 probe revealed 6-8 fragments in each strain (except for #619 in which there appears to be some artifact). Some fragments were present in all strains (including PG-2) whereas others were uniquely present or absent in a single strain. Analysis of the same DNA samples with the 0.6 kB and B-1 probes led to the same conclusions with respect to heterogeneity of the hybridization patterns. As expected, fewer fragments were revealed by these probes (3-4 in each strain with each of these two

probes), some fragments were shared and others were present in several or only one strain. Only 2 out of 8 strains had identical hybridization patterns, and the identity of the patterns was present with each probe tested. Strains showing a unique band with the *sig-1* probe (eg, #618 in Table 1) also showed a unique but different sized, fragment with 0.6 kB probe.

In this study ewe #627, was the only animal from which MA was isolated at each sampling time, secreting the organism for more than a year, until slaughtered. Hybridization patterns with the various probes, and analyses of the genomic rearrangements for MA isolates from ewe #627 are presented in BARD publication #. Isolation of MA from other animals in the experimental group occurred throughout the period, although not continuously. Data are summarized in the tabular form for two other animals, #611 (Table 2) and #617 (Table 3), from which MA was isolated up to the end of the trial but not at each sampling time. For comparison, the hybridization data for the prototype ewe #627 are also summarized in tabular form (Table 4).

Table 2: Band patterns revealed by the *sig-1* probe hybridization in DNA from MA isolated from ewe #611 at different sampling times

kB	PG2	Sampling Time								
		3	4	11	12	13	14	16	17	18
6.5		+								
6.0				+	+	+	+	+	+	+
5.3	+		+							
4	+		+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+
2.5	+									
2.2		+	+	+	+	+	+	+	+	+
2.0				+	+	+	+	+	+	+
1.8										
1.5										
1.3	+	(+)	+	+	+	+	+	+	+	+
1.1		(+)	+	+	+	+	+	+	+	+
1.0	+									
0.6			+							

The *sig-1* probe recognized 6-8 genomic fragments, a larger number than would be predicted on the basis of the *avg* locus as currently recognized. Some of these fragments are present in all MA isolates tested, whereas others are present at only one or two sampling times. In spite of the complexity of the genomic fingerprints with the *sig-1* probe, and equally complex patterns produced by use of the 0.6 kB probe, it was possible to focus on isolates at specific sampling times, in which genomic rearrangement was clearly occurring. For example, sampling times 4-6 and 13-14 in ewe #627 (highlighted in Table 4). This phenomenon is discussed in BARD publication #6. Less complex patterns were obtained with the *b-1* probe, recognizing 3-5 fragments. This probe also detected changes in size of genomic fragments indicative of genomic rearrangement in the *avg* locus, as described with the *sig-1* probe. The *a-1* probe recognized only one genomic fragment, located in the *avgA* gene, in all MA isolates tested. Major size differences were detected in this single fragment, making this gene a good candidate for tracking genomic rearrangement in the locus as a whole.

Table 3: Band patterns revealed by the *sig-1* probe hybridization in DNA from MA isolated from ewe #617 at different sampling times

kB	PG2	Sampling Time										19	20
		3	4	6	7	11**	12	15	16	17	18		
6.5									+				
6.0			+	+		+	+	+		+	+		+
5.3	+				+								
4.3		+										+	
4	+		+	+		+	+	+	+	+	+		+
3	+	+	+	+	+	+	+	+	+	+	+		
2.5	+												
2.2		+	+	+	+	+	+	+	+	+	+		+
2.0		+	+			(+)	+	+		+	+		+
1.8													
1.5			+	+	+	?	+	+		+	+		+
1.3	+		+	+	+	?	+	+		+	+		
1.1													
1.0	+												
0.6													

**Sample has less DNA and the pattern is hard to distinguish.

Table 4: Band patterns revealed by the *sig-1* probe hybridization in DNA from MA isolated from ewe #627 at different sampling times

[illegible]

On the basis of the genomic fingerprints of the *avg* locus we have attempted to establish whether the "reappearance" of MA in the milk was due to reactivation of latent infection in the animal or reinfection from infected flockmates. In both animals (#611 and #617) reappearance of MA was detected at sampling time #11 after negative results in sampling for about three and two months, respectively. It is possible that some secondary, environmental factor may play a role in the "reappearance" of MA in several animals at this particular time.

Summary of the hybridization data from this case study support the following:

1. MA isolates at sampling time 11 from both ewe 611 and 617 have the same genomic fingerprint with the *sig-1* probe. (See Tables 2 and 3).
2. The genomic fingerprint of this MA "*avg*-subtype" is not the same as the last prior isolate from the same animal.
3. This "*avg*-subtype" was present in two animals at the start of the study, including #617 itself. However in #617 another subtype appeared for several weeks before milk levels decreased and isolation attempts were negative.
4. This "*avg*-subtype" was detected in #611 at all subsequent sampling times (about 3 months).
5. In animal #617 this "*avg*-subtype" persisted or reappeared at several sampling times, although MA isolates with different genomic fingerprints were isolated at intermediate sampling times.

It remains to be established if this particular "*avg*-subtype" has properties which allow this MA strain to escape normal host defenses and to establish chronic asymptomatic infection. This will be investigated in the continuation of the project, using archived isolates and serum samples from all the animals.

A separate field case study was conducted in a closed sheep herd suffering a severe outbreak of Contagious Agalactia. Serum samples and MA isolates were obtained from seven diseased animals at weekly intervals for about 2 months, or until milk production ceased. No persistent infection was established in these animals. On the basis of preliminary and partial data, several trends are apparent:

1. In contrast to the marked heterogeneity in genomic fingerprints among MA isolates from chronically infected flockmates, *avg*-genomic fingerprints of MA isolates from diseased animals in the same flock were very similar to each other (outbreak representative fingerprint).
2. During the relatively short time frame of sampling in this case study, rearrangements in the *avg*-genomic fingerprint were not detected in the samples tested until now.

Analysis of archived samples will be continued with diagnostic tools developed on the basis of recent elucidation of the structure of the *avg*-gene family.

Data Appendix #3:

Antigen Capture ELISA Testing of Cattle Herds Differing in MB Status

Development of serological tests to evaluate exposure of dairy and beef herds to MB is a priority for the US. European studies using whole antigen ELISA have shown the value of this test for cleaning up herds (by aggressive culling) in geographic areas with incidence of 20% positive or less. With much higher seropositivity indices, the US environment requires discriminating tests that can be correlated with healthy carrier status. VMRI has approached this problem by devising antigen capture ELISA serology with three different monoclonal antibodies, and comparing the results with those obtained with a whole antigen ELISA. Eight herds totaling 1968 animals were available for serological investigation (Table 1). Three herds (herds A, B, C) were small isolated herds with no exposure to specific respiratory pathogens, and no history of isolation of MB from nasal swabs. One "herd" (herd D) was composed of 10 calves from one of the above herds, after double dose vaccination with an experimental formalin-inactivated MB vaccine prepared with 2 antigenically distinct strains of MB, both of which expressed VspA or VspC. Two herds (herds E and F) were large herds sampled shortly after they suffered significant outbreaks of MB mastitis, respiratory disease, and arthritis. These herds had recently purchased large numbers of cows, and only partial serological sampling of these herds was available. Two herds (herds G and H) were large herds with a history of MB detection two or three years prior to serological sampling, with repeated culture-negative bulk milk tank samples during the

year prior to serology, and with a history of no or minimal purchases of cattle. Only partial serological sampling of these two herds was available.

Table 1. Proportion of seropositive cattle in herds tested by ELISA.

Herd	Nr of cattle in herd	Whole antigen	MYB87 capture Ag-	MYB163 capture Ag-	MYB66 capture Ag-
A	28	2/20	6/20	7/20	2/20
B	20	3/20	1/20	4/20	1/20
C	8	3/8	1/8	6/8	1/8
D	10	6/10	9/10	4/10	0/10
E	1200	62/102	13/102	40/102	70/102
F	200	96/96	14/96	43/96	75/96
G	300	17/49	8/49	5/49	13/49
H	200	88/90	28/90	62/90	15/90

The ELISA tests were set up with a single dilution of serum tested on triplicate wells. Serum diluted 1:10 in PBS with 10% fetal bovine serum was used throughout and all sera to be tested were diluted and frozen as aliquots in template microplates prior to the assays. Animals were scored positive when they gave absorbance results exceeding the microplate negative value by two standard deviations of the mean. Three monoclonal antibodies were used for antigen capture with a Bryj-58 lysate of strain M23 of MB (the strain used to develop the monoclonal antibodies). Of the three monoclonal antibodies, MYB87 captured VspA, VspC, and a small unidentified Vsp. MYB163 captured a 70 kd antigen, and MYB66 captured a 19 kd surface protein. All of these captured proteins were previously shown to be specific to MB strains, although cross-reaction of MYB163-capture antigen and host inflammatory proteins is considered possible but yet unproven. Several important conclusions can be drawn from the data obtained. Antibody responses to VspA and VspC were not good as diagnostic indicators of acute (herds E and F) or chronic infection (herds G and H). In addition, cows from herd A were found reactive even though there was no cultural or clinical evidence of infection for that herd. Antibody responses to the 19 kd antigen captured by MYB66 may be more specific to MB than those detected with whole antigen or antigen captured by MYB163. The 19 kd antigen did not detect responses in vaccinated herd D, and gave very low proportion of responses in chronic herds (herds G and H).