The Humoral Immune Response of Elk (*Cervus elaphus nelsoni*) and Mice to Vaccination with *Brucella abortus* Strain RB51

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(ABSTRACT)

Vaccine *Brucella abortus* strain RB51, unlike the wild strain 2308 and another vaccine strain (strain 19) does not induce anti-O-chain antibodies. An efficacious vaccine strain that fails to produce an O-chain and thus a lack of an anti-O-chain humoral response greatly simplifies identification of vaccinated versus field strain infected animals. The three primary objectives of this research were the following: 1) to develop a serological assay to detect anti-RB51 antibodies in vaccinated elk (*Cervus elaphus nelsoni),* 2) to identify potential antigenic alterations in RB51 after vaccination of elk and BALB/c mice, and 3) to confirm the general stability of RB51.

Elk were divided into four groups based upon gender and the route of inoculation (subcutaneous or ballistic) of RB51 bacteria. This study developed a highly reliable ELISA (using a monoclonal anti-bovine IgG_1 antibody and acetone killed whole RB51 bacteria) which can identify RB51-vaccinated elk. Also, isolates recovered from RB51-vaccinated elk were inoculated into female BALB/c mice whose spleens were then cultured. All elk and mice isolates were bacteriologically, biochemically, and serologically evaluated.

This study showed that RB51 is a highly stable strain, which does not revert to smooth morphology or initiate synthesis of LPS-O-chain, maintains it biochemical characteristics, does not undergo detectable antigenic variations, and remains attenuated even after successive passages in elk and mice.

Overall, this research indicates that RB51 is a vaccine candidate for the prevention of brucellosis in elk. Further studies are needed to determine the protective capabilities of RB51 in elk.

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cfu	colony forming units
CMI	cell mediated immunity
ELISA	enzyme linked immunosorbant assay
Ig	immunoglobulin
IL	interleukin
INF	interferon
kDa	kilodalton
LPS	lipopolysaccharide
MW	molecular weight
PBS	phosphate buffered saline
PBST	phosphate buffered saline - Tween 20
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
% T	percent transmittance
TBS	Tris buffered saline
TBST	Tris buffered saline - Tween 20
TSA	trypticase soy agar
TSB	trypticase soy broth

Chapter One

Literature Review

General Characteristics of the Genus *Brucella*

The genus *Brucella* is comprised of aerobic, facultative intracellular, gram negative coccobacilli bacteria that replicate within host macrophages (54). These organisms are non-motile, non-spore forming, and often grow best in the presence of 5 to 10% CO_2 (3). The genus *Brucella* hypothesized to have evolved from an *Agrobacterium-Rhisobium* complex, a plant pathogen (42). The genus contains six species: *B. abortus, B. melitensis, B. suis, B. canis, B. ovis*, and *B. neotomae* whose DNA sequences share greater than 90% homology (62, 130). Organisms within these species are often subdivided into several biovars. Currently, B. *abortus* biovar 2 is believed to be the organism from which all other recognized *Brucella* organisms evolved (77).

Bacteria in the *Brucella* genus can be identified through a combination of bacteriological and biochemical tests. *Brucellae* appear as gram-negative coccobacilli upon gram staining. They are urease positive, citrate negative, indole negative, catalase positive, and oxidase positive (3).

Specific Characteristics of *B. abortus* Including Rough and Smooth Morphology

B. abortus was first described by Bang in 1897 (8). It currently infects thousands of animals and humans throughout the world causing large economic losses as well as suffering in both animals and humans (37, 124, 142). *B. abortus* bacteria can be differentiated from other species within the genus and classified into seven biovars by a combination of characteristics like dye sensitivity to basic fuchsin and thionin (50), CO_2 requirement, H_2S production, and the presence of A, M, or both surface antigens (3) and by bacteriophage typing (27).

Once an organism is identified as belonging to the *B. abortus* species, it can be further classified as having a smooth or rough morphology by detection of the respective presence or absence of "an O-polysaccharide [chain] attached via [a] core oligiosaccharide to the lipid A which anchors the molecule to the outer membrane" on the bacteria. (21). The O-polysaccharide (O-chain) of smooth *B. abortus* species is composed of linear homopolymers of 4,6-dideoxy-4-formamido-alpha-D-mannopyranosyl residues known as perosamine. (19, 20). Smooth *B. abortus* bacterium can be differentiated from rough bacterium by the smooth organism's lack of agglutination in a 0.1% solution of acriflavin (14), lack of absorption of a 0.05% crystal violet solution (133), and a positive reaction in a BRU 38 colony blot test which utilizes a monoclonal antibody (BRU 38) specific for O-chain (101). Rough *B. abortus* organisms (such as *B. canis)* will give opposite results in these tests.

In smooth *Brucella*, the LPS associated O-chain and other outer-membrane components interact primarily with the host's immune system (43, 46, 82, 96, 131). Researchers have capitalized on this fact by developing serological tests able to detect antibodies against the O-chain which would only be found in infections with smooth strains of *Brucella* (3, 83, 105). Four commonly used serological tests are the Buffered Brucella Antigen (BBA), the standard plate agglutination (SPT), the rivanol (Riv), and the complement fixation tests (CFT) (121). Although the CFT often is reported to be the most sensitive test, researchers usually run a combination of these tests for maximal sensitivity and specificity. Currently, a variety of ELISA tests have been developed which approach 85% specificity and sensitivity.

The O-chain of smooth *B. abortus* organisms is very similar to that of *Bordetella bronchiseptica, Campylobacter fetus, Moraxella spp.,* and *Yersinia enterocolytica* serotype *09* (3, 25) resulting in serological cross-reactivity between smooth *Brucella* organisms and organisms in the Enterobacteriaceae family (26). This cross-reactivity can result in serologically false positive results when testing animals for infections with smooth *B. abortus* bacteria (including both strain 19 vaccine and field strain bacteria) which were previously exposed to the Enterobacteriaceae family.

Truly rough strains of *Brucella* do not contain perosamine and thus should not produce an immunological response against LPS-associated O-chain. This lack of O-chain in rough *B. abortus* is very important as it allows for the differentiation of animals infected with rough or smooth organisms. To differentiate a serological response to either smooth or rough *Brucella* infection, two tests would need to be performed on each sample. One test would detect O-chain specific antibodies produced in response to infection with smooth strains. The second test would detect antibodies specific for antigens common to both rough and smooth *Brucella* organisms. Animals infected with a smooth strain of *Brucella* should produce antibodies against both O-chain and common antigens. Animals infected with rough strains would produce antibodies only to the common proteins. Ideally, for precise identification, infections with rough organisms would elicit antibodies to antigens only present on rough strains. Currently, the most sensitive and specific way to detect a *Brucella* infection is by culture. The presence of viable *Brucella* is sufficient to diagnose brucellosis, yet the lack of a positive culture does not rule out the disease since a very low number of bacteria may be present in an infected individual.

B. abortus Infections in Humans

In humans, infection with *B. abortus* causes a disease known as undulant fever, Malta fever, or Bang's disease. Infected humans can suffer protracted and debilitating symptoms most commonly including intermittent fevers, malaise, weight loss, back pain, joint pain, nervousness and depression. Physical findings can reveal lymphadenopathy, splenomegaly, and joint swelling while further investigation may reveal genito-urinary involvement, arthritis, spondylitis, osteomyelitis, meningitis, and/or endocarditis.

The first reported human brucellosis infection within the U.S. occurred in 1906 although infections likely occurred for centuries prior to this. Between 1906 and 1922, all known infected individuals had some association with goats or had consumed unpasteurized goat milk products such as cheese (142). Until this time, it was believed that human transmission occurred only through contact with goats but in 1924 a slaughterhouse worker who had no exposure to goats contracted the disease. As a result, physicians started looking for other human cases. In addition to transmission through contaminated milk, humans have also been shown to contract the disease through contact with infected fetal animal tissues, infected animal blood, and through laboratory exposure (142). The incidence rate of human *Brucella* infections increased to a high of 36.9 new cases per 100,000 individuals per year for the period from 1945-1949 (37). Since then, the incidence within the U.S. has markedly decreased largely due to milk pasteurization, the USDA's *Brucella* Eradication Program started in 1934, and public (farmer and hunter) education programs (37). While the number of reported human infections within the U.S. has decreased dramatically, some believe that as many as 26 unreported cases exist for every one diagnosed case (142).

Transmission, Pathogenesis, and Control of *B. abortus* Infections in Domestic Cattle

Establishment of a new *Brucella* infection in a susceptible animal depends upon the dose, exposure route, and virulence of the bacteria as well as the health of the animal host. Although some are naturally immune (48), ungulates are most commonly infected after exposure to infected

placentas, fetal tissues, or fluids which often contain very high concentrations of organisms and which the animals lick or consume soon after abortion or birth (2). Cattle are most frequently infected with *B. abortus* biovar one. Oral, intramammary, respiratory, venereal (11) and congenital transmission have been documented in domestic cattle (32, 98). Sexually mature pregnant cattle are more susceptible to infection than are immature cattle of either gender (34, 48). Susceptibility also increases both with pregnancy and stage of gestation (33, 34). After infection, *Brucellae* usually travel to the draining lymph nodes where they replicate and then disseminate through the blood to other tissues, most frequently the mammary gland, the reproductive tract, and associated lymph nodes (48). *B. abortus* preferentially proliferates in areas with high concentrations of erythritol such as the gravid uterus of ungulates, fetal tissues, and the male genital tract (110). In bovine adults, *Brucella* has also been cultured from bones, joints, eyes, and the brain. In bovine feti, *Brucella* is most often cultured from the stomach, lungs, and spleen (3). A high correlation has been shown between infection with *B. abortus* and hygromas of the knee (44).

Latent infections of *B. abortus* have been documented in cows which did not show a positive serological response until after parturition or abortion (45). Wilesmith estimates that approximately 3% of heifer calves born to infected dams possess latent infections, which will not be serologically detectable until their first parturition (135). Since these animals may be incorrectly classified as free of the bacteria in serological tests, this allows for a potentially unrecognized reservoir of the bacteria in a herd.

B. abortus infection in cattle may result in late term abortions, births of nonviable calves, retained placentas, hygromas, and sterility (36, 111). The exact cause of abortion in infected cows is not fully understood. Two theories have been proposed. The first theory suggests that the bacterial invasion and proliferation in epithelial cells of the chorion produces a placentitis and endometritis (48). This could cause impaired placental function which may prevent a free exchange of nutrients and wastes between the mother and fetus, eventually resulting in termination of the pregnancy. Contrary to this theory, abortion has been observed in many instances with only minimal placentitis. The second theory proposes that abortion results from the endotoxic effects of *Brucella's* LPS. Studies of lipopolysaccharides isolated from other gram-negative bacteria have produced highly variable placental changes and alterations in progesterone levels (a hormone necessary for the maintenance of pregnancy) (4, 94). Regardless of the mechanism, *Brucella* infection has been shown to cause high levels of fetal stress evidenced by elevated cortisol levels. As a result, placental hormone production shifts from predominately progesterone to estrogen with an associated increased synthesis of prostaglandin F2-alpha ($PGF_{2\alpha}$) by the endometrium resulting in premature delivery or abortion (48).

B. abortus infection stimulates the production of IgM, IgG_1, IgG_2, and IgA antibody isotypes detectable in both milk and sera of cattle (87). In sera, IgM is produced at high levels soon after infection but then quickly declines as production of IgG isotypes increases. Among cattle, IgG_1 is consistently produced at high levels in the serum following infection (89). IgG_1 has been shown to have a high affinity and specificity for the *Brucella* antigens in serological tests (89). Therefore, detection of elevated levels of IgG_1 specific for *Brucella* antigens may be valuable in detecting *Brucella* infections. In contrast, IgM has been shown to react nonspecifically in many serological tests causing high rates of false positive reactions. Therefore, detection of IgM would not be reliable in diagnostic tests (140).

Currently, the U.S. is attempting to eliminate brucellosis through a combination of quarantine, serological tests, and slaughter programs, and vaccination as directed by the Animal and Plant Health Inspection Service's National Eradication Program (129). Since the introduction of brucellosis into a previously non-infected herd is usually caused by the new addition of an infected animal, farms should quarantine new breeding animals until they have completed one gestation

period with no indications of infection. To prevent transmission between herds, animals shipped between states or into a certified *Brucella* - free herd should be tested for exposure to *Brucella.* Any animals which test positive should be culled and slaughtered. In situations where a large percentage of a herd has been exposed, a complete depopulation of the farm is recommended. This would eliminate not only the known infected animal but also animals which test negative but may still harbor the bacteria. Animals that are near the infected farms are vaccinated to increase the animals' immunity. Until recently, the general recommendation was to vaccinate only heifer calves between four and twelve months of age with a reduced dose of *B. abortus* strain 19 vaccine. Vaccination of older animals often results in persistent titers that interfere with testing for natural infection.

Vaccines: *B. abortus* strains 19 and 45/20

B. abortus Strain 19 was first isolated from a Jersey cow in 1923 and has been the USDA approved vaccine for use in cattle for many years. It is a naturally attenuated, semi-rough strain of *B. abortus* that multiplies within the animal for only a relatively short period of time. In vaccinated calves, antibody titers usually do not persist for more than 10 months (86). It differs from 2308 biovar 1 (the USDA challenge strain) by its inability to grow in the presence of the dye thionin blue (1:500,000), penicillin (5 U/ml) or erythritol (1 mg/ml) (15). Unfortunately due to mutations from the original seed stock, some vaccine lines of Strain 19 vary slightly in their sensitivity to these substances (15). When challenged with strain 2308, strain 19 confers an approximate 65–75% protection rate in cattle (73).

Strain 19 has the **distinct disadvantage** of promoting the production of O-chain specific antibodies which can be detected in serum and milk. Ideally, animals vaccinated prior to eight months of age will lose their antibody titer by eighteen months of age but this frequently does not occur. Animals vaccinated after eight months of age have an increased chance of maintaining titers for extended periods. These anti-O-chain titers interfere with serological tests thereby complicating disease diagnosis (85, 118, 139). Nielson et al (88) have developed indirect and competitive enzyme immunoassays that can differentiate some vaccinated from naturally infected cattle through the use of highly purified O-polysaccharide antigen. Like other popular tests, these tests utilize the fact that strain 19 is less virulent than 2308 and therefore most cattle clear strain 19 bacteria faster than 2308 bacteria. Since the strain 19 bacteria would be exposed to the host's immune system for a relatively short period of time and at relatively lower doses than encountered in natural infections with 2308, animals infected with strain 19 should not produce elevated levels of antibodies against the O-polysaccharide. In contrast, animals naturally infected with 2308 would produce significantly higher levels of anti-O-polysaccharide antibodies since these animals would be exposed to a large number of bacteria which would persist and multiply within the animal. Also due to the relatively fast clearance of strain 19 from vaccinated animals, they may only respond to the most readily available epitope on the O-chain (the 'tip epitope' exposed on the surface of the bacterium). On the other hand, naturally infected animals may have opportunity to produce antibodies to the 'tip epitope' on the outer-most surface of the bacterium and to epitopes along the length of the O-chain (the 'length epitope'). When purified O-polysaccharide antigen is used on a polystyrene surface common in both indirect and competitive immunoassays, the tip epitope binds directly to the polystyrene and thus is unavailable for interaction with anti-tip epitope antibodies. Length epitopes remain readily available. As a result, strain 19 vaccinated animals should demonstrate little to no reactivity with purified O-polysaccharide antigen while infected animals would strongly react. Although this test was often successful in differentiating vaccinated from naturally infected cattle, it could not accommodate vaccinated animals with persistent infections, animals vaccinated with high vaccine doses, or animals naturally infected with low numbers of bacteria.

In cattle, strain 19 can induce persistent infections with resultant shedding of organisms in milk and vaginal secretions (71). Persistent infections are more common in cattle vaccinated at greater than one year of age (69). Vaccination with strain 19 can also cause abortion, arthritis, or endotoxic shock in vaccinated animals (30, 107, 118). Strain 19 has also been shown to be pathogenic to humans (142).

In order to overcome the described difficulties with strain 19 vaccination particularly those associated with seroconversion, researchers have developed other vaccine strains. One of these is strain 45/20. Strain 45/20 is a rough mutant strain of *B. abortus* and is administered killed and mixed with incomplete Freund adjuvant (a water-in-oil-emulsion) to increase efficacy(86). While it does induce protection comparable to that of strain 19 after two vaccinations and annual boosters (not necessary with strain 19), and if prepared appropriately, does not stimulate anti-O-chain antibody production, it is less convenient to use. The vaccine mixture can also cause significant local tissue reactions in vaccinated animals which reduces carcass value. Also, strain 45/20 is has a high tendency to revert to smooth morphology which impedes its use as a vaccine. As a result, strain 45/20 vaccine is unsuitable for widespread use (139). Recently, *B. abortus* vaccine strain RB51 has been developed and has been approved for use in the U.S. as an alternative to strain 19 vaccination. It will be discussed in length later.

Epidemiology of Brucellosis in Cattle within the U.S.

Within the U.S., the number of *Brucella* infected cattle herds is steadily decreasing however, newly infected herds are still being identified. From September 1995 to September 1996, 142 new *Brucella* infected herds were identified in 16 states. One hundred and twelve cattle herds were depopulated during the 1996 fiscal year with an estimated cost of $3,059,270 in indemnities (93). As of September 1996, 41 herds in 8 states were known to contain infected cattle, down from 68 herds in 16 states in September 1995 (128). It is predicted that Brucellosis will be eradicated from domestic livestock within the U.S. by December 31, 1998 (93).

Unfortunately a re-introduction of the disease within the U.S. is possible as many countries still have a high prevalence of brucellosis in domestic livestock which could infect our herds through imports of infected animals. Also *B. abortus* bacteria have been isolated from many wildlife species (35, 78, 120, 121). Wildlife has the theoretical potential to transmit the infection to cattle especially in grazing areas where the animals occasionally commingle (123). Within the scientific community, the frequency of such transmissions is believed to be low. In fact, no cases of transmission from wildlife to domestic livestock under natural conditions have been confirmed. However many cattle producers perceive the risk to be substantial. As a result, some producers advocate the depopulation of infected or potentially infected wildlife herds. To avoid this, brucellosis in wildlife must be eliminated or at least controlled.

Characteristics of an Ideal Vaccine for Brucellosis

Although likely impossible to achieve, the characteristics of an ideal vaccine for a disease can be defined. Vaccines should be developed to most closely approximate this ideal. For prevention of *B. abortus* infections in both domestic and wildlife species, an ideal vaccine would have the following characteristics (107):

1) provide complete, long term protection for all vaccinated animals with a single inoculating dose.
2) not revert to a virulent form that may cause disease.

3) not produce disease or persistent infection regardless of the number of inoculations, the age, or the physiologic status of the animal.
4) not interfere in the diagnosis of infected individuals.
5) not produce disease in humans or other non-target species.
6) not cause the meat or milk of the vaccinated animal to be unsafe for human or animal consumption.
7) be safe (to both the vaccinator and the animal), be reliable, and be inexpensive to produce.

Use of Mice in Brucellosis Research

Mice have been used as the model for the study of brucellosis and *Brucella* vaccines for many years (9, 96, 119). Mice are relatively easy and inexpensive to house. Their immune systems have been extensively characterized through past studies (96). Most importantly, populations of genetically identical mice are available which allow researchers in labs throughout the world to work with genetically identical animals thereby eliminating much individual animal variation.

When compared to strain 19, strain RB51 inoculated into mice have been shown to be rapidly cleared from the host, to cause only minimal pathology, and to offer a significant level of protection against challenge with strain 2308. BALB/c mice inoculated with 1×10^9 cfu have been shown to clear an RB51 infection by four weeks post inoculation (106), whereas mice injected with only 3.7×10^4 cfu of strain 19 and mice injected with 3 to 6×10^6 cfu of 2308 still had bacteria recovered from their spleens at 6 weeks and 24 weeks post inoculation, respectively (79).

Pathologic lesions induced by vaccination with strain 19 or strain RB51 have also been shown to differ. In pregnant mice, Strain 19 bacteria can cause severe necro-suppurative placentitis and fetal death while very high doses of strain RB51 produce only a mild placentitis without fetal death (126). In mice, *B. abortus* strain RB51 has been proven to induce protective immunity against challenge with *B. abortus* (66, 106). But when compared to strain 19, strain RB51 affords a slightly lower resistance to infection and a less persistent antibody response to strain 2308 challenge in mice (115, 117). In cattle RB51 and strain 19 afford similar protective responses (23).

Both humoral and cell mediated immune responses (CMI) have been shown to participate in protective immunity to *B. abortus* in both mice and cattle (6, 68, 79, 95, 117). In mice, both strain 19 and 2308 organisms produce predominately IgG_{2a} and IgG_3 anti-O-chain isotype antibodies at four and six weeks post-inoculation (47). The passive transfer of specific anti-O-chain antibodies of the IgG_{2a} or IgG_1 isotype results in partial protection against challenge with low doses of strain 2308 (6, 79, 90, 95, 137). This antibody mediated protective effect has not been demonstrated in ungulates. The predominant isotype response to strain RB51 vaccination in mice has not been determined. However, it has been shown repeatedly that mice vaccinated with strain RB51 do not produce antibodies against the O-chain of LPS (108).

Araya et al (6) found that antibodies to O-chain provided significant protection to mice at three weeks PI, while T cells did not provide protection until four weeks PI. Transfer of both antibodies and T cells prior to challenge provided significantly higher levels of protection than either alone. In this study, both CD4 and CD8 subsets were found to provide equal levels of protection after passive transfer. Oliveira et al (90) believe that $CD8^+$ T cells are largely responsible for the success of the cell mediated immune response to *Brucella* infection based on their studies with MHC class I and class II knock-out mice. In this study, strain 2308 will colonize the spleens of mice unable to produce $CD8^+$ T cells in higher numbers and for significantly longer periods than spleens of $CD4^+$ T cell deficient or control mice. Also, $CD8^+$ deficient mice produced

elevated levels of IL-10 (a cytokine responsible for immunosuppression through control of macrophage function and IFN-gamma production) (53) and decreased levels of IFN-gamma (a cytokine responsible for the up-regulation of macrophage anti-*Brucella* activity) (65). These cytokine alterations may contribute to the higher susceptibility to *Brucella* infection. (90).

Characteristics of *B. abortus* strain RB51

B. abortus RB51 is a stable, rough mutant of strain 2308 which has been shown to induce protection against challenge with smooth virulent *Brucella spp.* in cattle, swine, mice, and goats (23, 57, 66, 136). The RB51 vaccine strain was produced by successive passages of the virulent smooth strain 2308 on trypticase soy agar containing rifampicin or penicillin (106). Examination of purified LPS extracted from strain RB51 by Western blot analysis with monoclonal antibody BRU 38 (specific for LPS-associated O-chain) suggests that strain RB51 does produce a minimal amount of LPS associated O-chain (106) although colonies are clearly rough when tested by colony uptake of 0.05% crystal violet (133) or agglutination in 0.1% acriflavin (14). No detectable levels were found with the BRU 38 colony blot enzyme-linked immunosorbant assay which utilizes monoclonal antibody BRU 38 specific for the O-chain (101, 105). Also, serological evaluation of animals repeatedly vaccinated with strain RB51 demonstrate no evidence of LPS associated anti-O chain antibody production (106,112, 117).

To date, strain RB51 has not been shown to revert to the more virulent smooth form after multiple passages in vivo (mice) and in vitro, suggesting that the organism is very stable (108).

RB51 has been shown to induce protective immunity to *B. abortus* challenge in both mice (66, 106) and cattle (23). In cattle, RB51 has been shown to produce a roughly equivalent level of protection against a challenge with *Brucella* field strain 2308 organisms as does strain 19. In adult dairy cattle, RB51 may be cultured from draining lymph nodes after vaccination with 1×10^9 to 1×10^{10} cfu of strain RB51 for several weeks (19) however, some adult and many calves younger than 8 months may clear the infection within two weeks (18, 22)

A major advantage of strain RB51 vaccination (regardless of the frequency, dose, or route of inoculation) is its inability to induce anti-LPS associated O-side chain antibodies which would interfere in the diagnosis of field strain infections of *Brucella* (18, 22, 23, 66, 106, 112, 116, 117). Although strain RB51 vaccination has been shown to cause a mild level of placentitis in mice after inoculation of high doses (126) and has been shown to produce in vitro cytotoxic effects on bovine chorioallantoic membrane explants (103), it has not been shown to induce fetal death or abortions in mice, goats, and cattle (18, 22, 49, 101, 106, 126). Of 826 cattle vaccinated with strain RB51 during pregnancy, only 1 aborted due to strain RB51 (Palmer, M.V., S.C. Olsen, and N.F. Cheville. Safety and Immunogenicity of *B. abortus* strain RB51 Vaccination in Pregnant Cattle presented at the Brucellosis Research Conference, Chicago, IL, November 9, 1996).

Serological differentiation of *B. abortus* infection from vaccination

In the past, researchers have utilized combinations of the buffered Brucella antigen (BBA), complement fixation (CFT), standard plate agglutination (SPT), and the rivanol (Riv) tests to identify animals infected with smooth strains of *B. abortus* (121). These tests detect antibodies to the O-polysaccharide found in smooth *B. abortus* and numerous members of the Enterobacteriaceae family. Unfortunately, no one test is both sensitive and specific enough to detect all infected animals and still maintain a low rate of false positives (72, 121). In fact, even when using these tests in combination, it is sometimes difficult to determine the true antibody status of the animal. The gold standard method of confirming *Brucella* infections is culture of the bacterium.

This method can only detect current infections and therefore could not suggest past exposure history. Since only a few organisms can be present in tissues not easily sampled, culture of multiple tissues at necropsy would be needed to suggest a lack of infection. Therefore, this method would not be useful for routine testing.

In an attempt to produce a more sensitive, specific, and standardized test that could be easily performed and interpreted on readily acquired samples, researchers have attempted to develop enzyme-linked immunosorbant assays (ELISAs) to detect antibody production in response to *B. abortus* infection (88). An ELISA is an immunologic test which can, depending on its configuration determine the level of antigen or antibody in a liquid sample. Two common types of tests are the indirect and the competitive ELISAs. Briefly, in an indirect ELISA, a known amount of antigen is bound to a multi-well polystyrene plate. A sample (usually sera or milk) is diluted and incubated in the antigen coated wells. After a period during which antibodies within the sample should have bound to their specific antigen, any unbound antibody is washed away. Next, a solution of a developing reagent (a marker molecule (eg. horseradish-peroxidase) conjugated to an immunoglobulin specific for the antibody of interest in the sample) is incubated in each well. After incubation, unbound conjugate is washed away and a substrate/chromogen solution is added which results in color development through the substrate by the enzyme conjugated to the immunoglobulin. The degree of color development in each well is assessed with a spectrophotometer and is directly proportional to the level of antibody in the test sample. A competitive ELISA differs in that after a known amount of antigen is bound to the plate, combinations of antibody solutions are added. These combinations consist of the unknown level of antigen-specific antibodies present in the sample and varying known concentrations of antigen-specific enzyme labeled antibodies. Both antibodies will compete to bind with the limited number of antigens bound to the plate. The amount of binding of the known concentrations of labeled antibodies can be used to derive an inhibition curve from which the level of antibody in the sample can be estimated. A high level of binding of labeled antibodies would suggest a low concentration of antibodies in the sample.

Researchers have attempted to develop ELISAs to detect antibodies against smooth *B. abortus* both in milk and serum (59, 88). ELISAs are valuable tests as they can often be automated and therefore capable of quickly evaluating a large number of samples. Also, ELISAs can be standardized such that identical tests can be run throughout the country. Lastly, since ELISA results are objectively reported in optical density values by an automated plate-reader, results need not be subjectively assigned by the researcher. However, there is great controversy involving the analysis of ELISA results. Many methods for interpreting ELISA data have been described (55, 59, 89).

Three methods of data analysis that appear frequently in the literature involve specific optical densities (OD), straight comparisons between pre- and post-inoculation samples, and percent positivities (89). Each of these methods have major limitations. Specific optical densities (calculated as OD of a post-inoculation test serum - OD of the pre-inoculation test serum) do not provide for inter-plate variability. As a result, only samples tested on the same plate can be compared thus precluding use in large study groups. To analyze the specific optical densities, most investigators assign an arbitrary value above which a sample would be considered positive. This value usually has no statistical validity.

Straight comparisons between pre- and post- inoculation values do not allow for comparisons between animals or between plates. Often negative cutoff values are chosen arbitrarily or are based upon a single sample per animal (for example the average plus three times the standard deviation of multiple duplicate wells of the animal's pre-inoculation serum sample). Straight comparisons also do not control for day-to-day variations.

Percent positivity is a ratio of the specific OD of the sample to the OD of a positive control. It is designed to represent where a sample lies along a straight continuum with zero binding of antibody being equivalent to 0% positivity and the antibody binding from the positive control being equivalent to 100% positivity. Therefore, the percent positivity of each sample represents the ratio of specific antibody in that sample to the amount of specific antibody in the positive control.

As mentioned previously, Nielson et al. (88) have developed a competitive ELISA to detect *Brucella* anti-O-antibodies in cattle with 85% accuracy. However, despite numerous attempts, a reliable serological test has not been developed to accurately, easily, and objectively distinguish strain RB51 vaccinated animals from non-vaccinated animals. Truly rough strains of *Brucella* do not contain perosamine and thus should not produce an immunological response against LPS associated O-chain. This lack of O-chain in rough *B. abortus* is very important as it allows for the differentiation of animals infected with rough or smooth organisms. To differentiate two tests would need to be performed on each sample. One test would detect O-chain specific antibodies produced in response to infection with smooth strains. The second test would detect antibodies specific for the antigens common to both rough and smooth *Brucella* organisms. Animals infected with a smooth strain of *Brucella* should produce antibodies against both O-chain and common proteins. Animals infected with rough strains would produce antibodies only to common proteins.

Strain RB51 vaccinated animals could be identified by culture and bacteriological and biochemical analysis of isolates (106), western blot analysis of serum samples, and dotblots (92). However, all of these tests are time consuming, expensive to perform, and proved to be of low sensitivity. Diagnosis of live animals by culture is not practical as it would only identify animals with organisms currently in their blood or milk and would not be able to identify vaccinated animals which cleared the bacteria. Both the Western blot and dotblot as well as some bacteriological and biochemical tests of cultured bacteria require subjective interpretation of results by the researcher. This subjectivity decreases the accuracy of the tests when performed in many labs by many different individuals.

Brucellosis in Wildlife

Bison (*Bison bison*) was the first wildlife species shown to be infected with *B. abortus* originally by serology in Yellowstone National Park in 1917 (78) and then by bacterial culture on the National Bison Range in 1930 (35). Since then, numerous studies have shown a high serological reactor rate in bison both in the U.S. and Canada (24, 52, 81, 102, 120, 127). In a 1989 study, greater than 50% of all Yellowstone National Park bison tested were serologic reactors against *Brucella* antigens (52). Davis et al. (40) demonstrated that the pathologic results of *B. abortus* infection in bison do not differ substantially from that in cattle. They also demonstrated that infected bison penned with susceptible cattle are as likely to transmit the disease as are similarly penned infected cattle. Currently, an estimated 4,400 bison with a 50% prevalence rate of *Brucella* infection are believed to inhabit the Greater Yellowstone area (Yellowstone National Park, Grand Teton National Park, and adjacent area (93). Use of the *B. abortus* strain 19 vaccine has been tested in pregnant bison cows and has been shown to confer only 39% protection against infection when compared to non-vaccinated control bison. In addition to a low level of protection, strain 19 was shown to produce "persistent antibody titers, vaccine induced abortions, and chronic strain 19 infections" (41).

To help prevent the spread of brucellosis from the bison herd to surrounding cattle herds (as well as to reduce the consumption of cattle feed by wondering bison), all bison which travel outside of the park boundaries are euthanized and tested for *Brucella* infection. Although this practice has been complicated by numerous legal battles, the slaughtered bison do serve as a

valuable indicator of the level of *Brucella* infection within the park's herd. Studies are in progress to evaluate the feasibility and efficacy of vaccinating bison for brucellosis.

Other ungulates tested in the U.S. for exposure to B. *abortus* include reindeer and caribou (*Rangifer spp.*), numerous deer species, moose (*Alces alces*) and pronghorn antelope (*Antilocarpa americana*) (39). Although *Brucella* organisms have been isolated from reindeer and caribou in Alaska since 1939 (63), all classified isolates have been identified as *Brucella suis* biotype 4. To date, no *Brucella* isolates have been recovered from pronghorn antelope in the U.S. Based upon serological evaluation, only one animal has been reported infected (1, 67). Although a large number of feral swine have been shown to be infected with *Brucella* primarily in the southeastern U.S. (138), most isolates have been identified as *Brucella suis* biotype 1 (10). As a result, reindeer, caribou, and feral swine do not appear to be important wildlife reservoirs for *B. abortus* in the U.S. at this time.

Very few mule deer (*Odocoileus hemionus*) and white-tailed deer (*O. virginianus*) have reacted in serological assays for *Brucella* (39) despite testing of thousands of animals. In addition only one *B. abortus* isolate has ever been recovered from a free-ranging white-tailed deer (28). It is believed that these two deer species do not play a role in the spread of brucellosis in the U.S. However, these populations should continue to be monitored as they are capable of being infected with *Brucella* under experimental conditions (7, 141).

After extensive testing, *Brucella* organisms have been isolated from only a few moose (29, 51, 64). Clinically, these animals were observed to be weak and emaciated. Severe pericarditis, myocarditis, lymphadenopathy, and peritonitis were common findings on necropsy. Since these animals are largely solitary animals (not forming herds) transmission within the population is believed to be rare at most. Therefore moose are not likely a reservoir for the disease. In fact, due to the weak and emaciated condition of infected animals it is believed that moose serve as a dead end host for *Brucella* (31).

Small mammals (rats, mice, raccoons, and opossums) may also be infected with *B. abortus* (80, 104). Rats, mice, and opossums may also act as transport hosts, ingesting infected material and then excreting *Brucella* in their feces or urine. Recovery of organisms from these species have only occurred after cattle herds in the area became infected. No evidence has been presented to show that these transport hosts are capable of causing infection in non-infected cattle herds (13).

B. abortus has also been isolated from coyotes (*Canis latrans*), domestic canines (*Canis familiaris*), and red foxes (*Vulpes vulpes*) in the U.S. (38, 61). These and other carnivorous mammalian scavengers are likely to be infected through ingestion of infected placentas and fetal tissues. As with other non-ungulant species, prevalence of the disease within the *Canis spp.* has been shown to increase soon after introduction of infected cattle to the area (38, 76). Domestic canines have been shown to shed *Brucella* organisms in vaginal and uterine discharges, urine, and feces (81,125). As a result of their shedding patterns and the fact that canines can travel large distances in a short period of time, canines should be further evaluated to assess their role in *Brucella* transmission (122) and to explore their use as sentinel animals (39)

Even insects have been shown to act as a host for *Brucella* organisms. Cheville et. al. (22a) found that the face fly (*Musca autumnalis*) which preferentially feeds on cattle birth fluids and tissues can act as a transport host of *B. abortus* depositing infected feces into the eyes of cattle. However no evidence has been presented to prove that the flies transmit brucellosis between cattle in natural conditions (22a).

Ecology of Rocky Mountain Elk

Elk have received considerable attention due to their involvement with brucellosis. Elk (also known as Wapiti) that occupy the National Elk Refuge belong to the North American elk subspecies *Cervus elaphus nelsoni.* Their range from at least the early nineteenth century have included the Rocky Mountains and mountain ranges from the fifty-fifth parallel southward to the thirty-sixth parallel (16). Numerous animals have been translocated to other parts of the continent (Texas, Oklahoma, Michigan, Pennsylvania, California, etc) usually to establish herds for hunting clubs. Currently, many small populations are scattered throughout the midwestern and eastern portions of North America (16).

Elk form a matriarchal society. Outside of the breeding season, immediate family units (composed of adult cows and their newborn to approximately three year old progeny) usually congregate in constantly migrating groups with frequent free movement of animals and/or family units into and out of the group. Bull elk at this time are either solitary or form bachelor groups (109). During the breeding season of approximately August until December (132), elk form harems in which one dominant bull elk pairs with multiple cows and their progeny. These groups are also very dynamic in animal composition (109). Between May and June, after a gestation of 247–262 days, cows isolate themselves from the herd for approximately one week for parturition. Calves may suckle their dams for up to nine months after which the calf may remain with its dam until sexual maturity (132). Female elk reach sexual maturity at two years of age, while males reach sexual maturity at three to four years of age (17).

The Origin of Brucellosis in Elk

The first serological evidence of *Brucella* infection was found in Yellowstone National Park elk (*Cervus elaphus nelsoni*) in 1932 by Rush (102). Serologic evidence has indicated the presence of *Brucella* in the Yellowstone bison population since 1917. It appears bison in Yellowstone contracted the disease when they were pastured with infected cattle and horses (75). Yellowstone elk probably were infected when they were wintered on common feeding grounds with infected bison and cattle. (75). However, elk from the Jackson Hole, Wyoming area (south of Yellowstone Park) were most likely infected independently from the Yellowstone elk population by cattle on ranches that took over the elk's winter ranges.

Numerous studies have estimated the level of infection in elk since it was first diagnosed (1, 29, 74, 121, 127). In 1978, Thorne et al. (121) estimated a 50% prevalence rate of *Brucella* reactors among mature cows. This study also indicated that the prevalence rate of *Brucella* infection in adult female elk tends to increase with the cow's age. Currently, 80,000 elk are believed to inhabit the Greater Yellowstone area (93).

In the past, researchers have utilized combinations of the buffered *Brucella* antigen (BBA), complement fixation (CFT), standard plate agglutination (SPT), and the rivanol (Riv) tests to help diagnose infected elk (121). However, even when utilizing multiple tests, infected animals may be incorrectly classified (84).

In elk, brucellosis has been shown to cause abortions, birth of premature and nonviable calves, hygromas and synovitis of calves as well as lymphadenopathy in all ages. Organisms have been cultured from many sites in both adult and calf elk including birth fluids, vaginal exudates, epididymes, seminal vesicles, ampullae, blood, and multiple lymphoid tissues (80, 122).

In one study by Thorne et al (122), a dose of 7.5×10^6 cfu of *B. abortus* strain 2308 was sufficient to cause infection (verified by culture) in all 27 mature elk (24 cows and 3 bulls) inoculated. The incubation time (days from infection until abortion) was 68-125 days. When housed with these artificially infected animals in small areas with similar densities to those found on

elk winter feed grounds, 17 of 18 mature elk cows and 6 of 6 elk bulls were found to be infected with *B. abortus. Brucella* was recovered at necropsy from one artificially infected cow fifty-six months after infection. Bacteria was intermittantly cultured from her blood until 68 weeks post-infection (122) however she maintained a titer against strain 2308 until necropsy. Five infected elk apparently cleared the infection.

Calves born to infected mothers had highly variable responses (122). Some calves were negative in serological tests but were culture positive at necropsy. Eighty-nine percent of calves born to infected cows possessed serotiters to *Brucella* at birth; however, the titer in many of these calves decreased and eventually disappeared. One calf supported an active infection until she was euthanized at four years. Of the twenty-nine calves maintained in the study after six months, 17% became infected between twelve and twenty-four months of age, 21% became infected between six and twelve months of age, 34% were not infected when removed from the study between eight and twelve months of age, 21% were not infected when removed from the study between twelve and twenty-four months of age, and 7% did not demonstrate an active infection until three years of age.

Studies have shown that infected elk can transmit the disease to susceptible cattle when all animals are maintained at a high density within a fenced pen (123). Currently, transmission from infected elk to cattle has never been documented under field conditions. Transmission between these species is believed to increase with increasing animal density and with exposure to aborted tissue and fluids (123). Although elk and cattle do not chose to mingle closely under normal field conditions and although elk tend to seek seclusion prior to calving (56), high animal densities might occur on common winter and spring feeding grounds thus concentrating infected and susceptible animals of both species (123).

History of the National Elk Refuge

The National Elk Refuge in Jackson Hole, Wyoming was established in 1912 in response to public outcry regarding elk consumption of livestock hay reserves and the starvation deaths of thousands of elk as a result of harsh winters and displacement from historical grazing areas (134, 100). Starting with only 1,760 acres in 1912, the refuge now consists of 24,000 acres and is administered by the U.S. Fish and Wildlife Service, Department of the Interior. The goal of the refuge is to provide supplemental feeding to elk in the winter only during periods of decreased forage availability and decreased grazing movement due to heavy snow cover. Typically, elk forage on the refuge grounds for approximately six months per year (November–May) and are fed a combination of hay and alfalfa pellets for approximately two months (February–March) based upon forage availability and snow depth (100, 134). Through management practices, the herd is kept at a maximum of approximately 7,500 head of elk.

Chapter Two

Serological Response of Elk Inoculated with *Brucella abortus* Strain RB51 and Characteristics of their Vaccine Isolates.

INTRODUCTION

The experiments described in this chapter were designed to evaluate both the cultural and serological response of elk vaccinated with *B. abortus* strain RB51. This evaluation is necessary to assess the potential value of *B. abortus* strain RB51 as a vaccine strain for the prevention of *Brucella* infection in elk. Isolates were cultured from RB51 vaccinated elk and characterized to ensure that *in vivo* passage of RB51 in elk did not alter essential characteristics of this bacteria. Also, ELISAs and dotblots were examined in an attempt to develop a serological test able to consistently identify RB51 vaccinated elk. Finally, an attempt was made to characterize the antigens of RB51 using Western blot analysis.

The ballistic route of vaccination was evaluated as it may be one of the most practical means of vaccination for wildlife.

METHODS AND MATERIALS

Experimental Design

Sixty- four elk (*Cervus elaphus nelsoni*) were captured from the National Elk Refuge in Jackson Hole, Wyoming and housed under the direction of Thomas Thorne DVM, PhD at the Sybille Research Center of the University of Wyoming. Elk were held in a total of six pens with saline control animals mixed with subcutaneously and ballistically inoculated elk. All animals were fed a high quality alfalfa hay and pellets. All elk were shown to be free of *Brucella* infections by five consecutive negative reactions in the buffered *Brucella* antigen (BBA), the standard plate agglutination (SPA), rivanol (Riv), and the complement fixation tests (CFT) (121).

The elk were divided into four groups based on the route of inoculation of viable RB51 organisms. The routes of inoculation and the dosage administered in each group is listed in Table 2.1.

TABLE 2.1. Elk experimental groups

Experimental group:	RB51 dose (cfu):	Elk identification numbers:
Female saline control	0	2, 5, 8, 11, 24, 31, 33, 50, 53, 55, 58, 61, 63, 66, 74, 82
Female ballistically inoculated	2×10^7 to 3×10^8	4, 25, 27, 30, 37, 40, 41, 46, 47, 49, 54, 59, 67, 69, 70, 72, 76, 79
Female SC inoculated	1×10^9	7, 9, 38, 39, 43, 45, 48, 56, 57, 60, 62, 68, 71, 75, 77, 78, 81, 89, 91, 99
Bull SC inoculated	1×10^9	3, 6, 10, 20, 22, 23, 32, 36, 84, 85

Ballistic inoculums were obtained from the National Veterinary Services Science and Technology division of the Animal and Plant Health Inspection Service (Ames, IA) which prepared biodegradable lactose-based pellets using the same culture of RB51 used for subcutaneous inoculation. The organisms used for ballistic inoculation were grown in a fermentor previously used to grow smooth *Brucella* organisms. Elk were bled on the following days:

TABLE 2.2. Elk serum sample collection schedule

Date of sample collection:	Days post inoculation:	Approximate weeks post inoculation
4-27-95	prevaccination	
5-10-95	0 - day of vaccination	
6-6-95	27	4
7-7-95	58	8
7-19-95	70	10
8-15-95	97	14
9-11-95	124	18
11-15-95	189	27

Blood was cultured at the Wyoming State Veterinary Diagnostic Laboratory, University of Wyoming (Laramie, WY) for the detection of *Brucella* for the first four months of the experiment.

One bull elk was necropsied every two months after inoculation; cows were maintained for future challenge experiments. However, one female elk (#38) was injured during handling and was subsequently euthanized and necropsied. At necropsy, the following tissues were cultured at Wyoming State University on *Brucella* media (3) and incubated at 37˚ C with and without 10% supplemental CO_2:

TABLE 2.3. Elk tissues cultured at necropsy

biceps femoris	left and right prefemoral l.n.
bronchial lymph node (l.n.)	left and right prescapular l.n.
cervix	prostate
left and right external iliac l.n.	left and right popliteal l.n.
left and right internal l.n.	spleen
liver	left and right supramammary l.n.
mediastinal l.n.	left and right suprapharyngeal l.n.
left and right mandibular l.n.	udder
left and right retropharyngeal l.n.	uterus
mesenteric l.n.	any visibly swollen joints

Seventeen elk isolates were recovered by the University of Wyoming staff. However, only ten of these isolates were found to be viable upon receipt in our lab on 28 June 1996.

TABLE 2.4. Date and tissue origin of elk isolates

Elk #:	Experimental group:	Date isolated:	Tissue origin of isolate:
3 *	Bull SC inoculated	09/31/95	mesenteric lymph node
20	Bull SC inoculated	05/23/95	blood
20	Bull SC inoculated	12/11/95	ampulla
20	Bull SC inoculated	12/11/95	prostate
20	Bull SC inoculated	12/11/95	left seminal vesicle
22 *	Bull SC inoculated	06/06/95	blood
22 *	Bull SC inoculated	09/18/95	ampulla
22 *	Bull SC inoculated	09/18/95	prostate
22 *	Bull SC inoculated	09/18/95	left seminal vesicle
23	Bull SC inoculated	06/06/95	blood
36	Bull SC inoculated	05/23/95	blood
38	Female SC inoculated	06/06/95	spleen
54 *	Ballistically inoculated	06/06/95	blood
62	Female SC inoculated	06/06/95	blood
69	Ballistically inoculated	06/20/95	blood
76	Ballistically inoculated	06/20/95	blood
77 *	Female SC inoculated	05/23/95	blood

* non-viable isolates upon arrival.

Identification of Elk Isolates as *Brucella spp*.

Upon receipt, each isolate was subcultured on a fresh trypticase soy agar (TSA) plate to assure culture viability and purity. All viable cultures were tested to ascertain that the organisms were of the *Brucella* genus by the tests listed in Table 2.5 using appropriate controls:

TABLE 2.5. Biochemical characterization of *Brucella* elk isolates

Test:	Positive Control: *	Negative Control: ^
Gram stain	none	stock Brucella strain RB51
Lysis in 3% KOH	stock Brucella strain RB51	E. Coli
MacConkey agar	Enterobacteriaceae aerogenes	stock RB51
Christensen's urease test media	stock Brucella strain RB51	E. Coli
Simmon's citrate test media	E. aerogenes	stock Brucella strain RB51
Oxidase SpotTest (Difco - Detroit, Michigan)	stock Brucella strain RB51	E. aerogenes
Indole SpotTest (Difco - Detroit, Michigan)	E. aerogenes	stock Brucella strain RB51
Catalase test (3% H_2O_2)	stock Brucella strain RB51	none

* positive controls: organisms known to react in the test.
^ negative controls: organisms not reactive in the test.

Identification of Elk Isolates as *Brucella abortus* strain RB51

All isolates were further evaluated by the following tests to determine if they were *Brucella abortus* strain RB51:

- ◊ agglutination in 0.1% acriflavin (14)
- ◊ uptake of 0.05% Crystal violet dye (133)
- ◊ growth on 0.0125% and 0.0250% rifampicin plates (Sigma - St. Louis, MO) (106)
- ◊ BRU 38 Colony Blot Test (101)

Standard stock cultures of *Brucella abortus* strains RB51 and 2308 from the Brucella isolates from the collection of Dr. Gerhardt Schurig were used as controls.

RB51 Whole Cell Antigen Preparation for Use in Elk ELISAs and Mice and Elk Dotblots

A lyophilized stock culture of RB51 organisms was reconstituted with sterile trypticase soy broth (TSB). Five hundred microliters of the broth solution was spread on a trypticase soy agar plate and incubated for 48 hours at 37˚ C in a humidified incubator supplemented with 5% CO_2. Roughness of the culture was verified by testing for agglutination in a 0.1% solution of acriflavin in distilled water (14) and by colony uptake of 0.05% crystal violet dye (133). After verification, the bacteria was subcultured on ten TSA plates and incubated for 72 hours (humidified 37˚ C chamber with 5% supplemental CO_2). The bacteria were harvested with approximately 3-4 ml of sterile distilled water, mixed with an equal volume of acetone, and stirred for three hours to kill the bacteria. The bacteria was then washed three times with sterile distilled water, pelleted by centrifugation at approximately 6000g at four degrees C for 10 minutes. The killed bacteria were resuspended in sterile distilled water to form a thick solution and aliquoted into sterile glass wheaton vials. The vials were frozen at -70˚ C for 24 hours and lyophilized.

ELISA to Detect Anti-RB51 Elk Antibodies

Lyophilized RB51 was reconstituted in sterile distilled water to 5% transmittance at 525 nm to form an antigen stock solution which was stored in polypropylene tubes at 4˚ C for no longer than one week. Immediately before use, this stock solution was centrifuged (3 minutes at 11,800g) , the supernatant discarded, and the original centrifuged volume of killed RB51 diluted 1:20 (v/v) in bicarbonate phosphate buffer (pH 9.6) thus making an antigen working solution. Appropriate wells of a 96-well medium-binding polystyrene plates (Costar - Kennebunk, ME) were coated with 200 ul of antigen working solution, the plate sealed with a parafilm sheet, and then incubated in a humidified chamber at 4˚ C overnight.

The plates were allowed to equilibrate to room temperature in a dark, humidified chamber and were washed four times with freshly prepared phosphate buffered solution containing 0.05% Tween 20 (PBST-20 - please see appendix for formulation for all buffers and reagents used in this thesis) using a semi-automated plate washer (NUNC Immunowash - Naperville, IL). For each washing, wells were filled with approximately 250 ul of PBST-20, allowed to soak for approximately 20 seconds, and then emptied by suction. After the fourth washing, the plates were inverted and tapped on a countertop to fully empty the wells. However, the wells were never allowed to completely dry as this can cause high non-specific binding (89). Serum samples were diluted 1:50 and 1:100 in PBST-20 in a 96-well polypropylene plate (Costar - Kennebunk, ME) immediately before dispensing. Two hundred microliters of each diluted serum sample were then incubated in the RB51-coated polystyrene plates at 37˚ C for 30 minutes. All incubations in future

steps were also performed at 37˚ C. After incubation, the wells were washed four times. Each well was then incubated at 37˚ C with 200 ul of a 1:6000 (v/v) dilution of mouse monoclonal anti-bovine IgG_1 (Veterinary Medical Research and Development (VMRD) BIg715A - Pullman, WA) in PBST-20 for 30 minutes. Bovine IgG_1 antibodies have been shown to have approximately 100% cross-reactivity with elk IgG_1 antibodies (60). Next, the plates were washed four times and then incubated for 30 minutes with 200 ul of polyclonal horseradish-peroxidase-conjugated goat IgG fraction to mouse IgG whole molecule (Cappel - West Chester, PA) diluted 1:800 (v/v) in PBST-20. The plates were washed four times. A developing solution was prepared immediately before use by mixing 10 mg of ortho-phenylenediamine (Sigma - St. Louis, MO) in 1 ml of 100% methanol with 100 ml distilled water and 100 ul of 30% H_2O_2. The wells were then incubated in the dark with 200 ul of developing solution at room temperature on a shaking platform (120 RPM) (Baxter Scientific) for 30 minutes. The developing reaction in each well was stopped with 40 ul of 0.18 M H_2SO_4. The optical density of each well at 490 nm was determined by a 96-well plate reader (Molecular Devices)

Elk ELISA Controls:

All serum sample dilutions were tested in duplicate wells to help assess inter-well variability. Positive and negative control wells were run in each plate. RB51 coated wells incubated with a bovine serum (Steer 66) with high anti-RB51 antibody level were designated as positive controls. All Steer 66 serum used was obtained from this one animal during one bleeding. To create identical positive control samples, a large volume of the bovine serum was diluted, aliquoted, and frozen at -70˚ C. A new aliquot was thawed and used in each plate. Steer 66 serum was arbitrarily diluted 1:100 in PBST-20.

Negative control wells consisted of the following:

- Pre-inoculation serum samples: two serum samples obtained from each elk prior to vaccination with RB51 or saline.
- 'No antigen' wells: two wells for each serum sample diluted 1:50 which were not coated with the RB51 antigen stock solution. Instead, these wells were incubated overnight with 200 ul of the bicarbonate phosphate buffer only.
- 'No serum' wells: two wells containing all appropriate reagents except serum. Two hundred microliters of PBST-20 were used instead of the diluted serum.

Wells containing all appropriate reagents except conjugated antisera were not tested on each plate as this combination had not been shown to cause nonspecific binding in previous ELISAs performed by the author.

Dotblot of Elk Samples

All reagents were equilibrated to and all incubations performed at room temperature. An antigen stock suspension of RB51 was prepared by diluting lyophilized, acetone-killed whole cell RB51 to 20% T at 600 nm in Tris buffered saline solution (TBS) (see appendix for formulation). The suspension was stored at 4˚ C in polypropylene tubes for no more than seven days.

An appropriately sized nitrocellulose sheet (0.45 um pore size - Micron Separations Inc. - Westborough, MA) was moistened in TBS, sandwiched within a 96-well Bio-dot (Bio-Rad - Hercules, CA) dotblot apparatus, and aspirated by vacuum. Each well was then washed with 100 ul of TBS and antigen coated by adding 30 ul of a 1:5 v/v dilution of the RB51 stock suspension in TBS. After 30 minutes, wells were vacuum aspirated to remove any visible liquid and then

incubated for 30 minutes with 100 ul of 0.25% Teleostean (fish) gelatin (Sigma - St. Louis, MO) in TBS. Wells were vacuum aspirated and washed 3 times with 100 ul of TBS, then 30 ul of serum sample (diluted 1:20 and 1:100 v/v in TBS - 0.25% fish gelatin) were applied to appropriate wells in duplicate and incubated for 30 minutes. After vacuum aspiration, five washings with 100 ul of TBS - 0.25% fish gelatin, 100 ul of mouse monoclonal anti-bovine IgG1 (Veterinary Medical Research and Development, Inc. - Pullam, WA) diluted 1:2000 v/v in TBS - 0.25% fish gelatin was added to each well for 30 minutes and each well was then washed five times with 100 ul of Tris-buffered saline with 0.05% Tween 20 (TBST) (see appendix for formulation). Horseradish peroxidase-conjugated goat polyclonal anti-mouse IgG (whole molecule) (Cappel -Durham, NC) was diluted 1:500 in TBS - 0.25% fish gelatin and 100 ul was added to each well. After thirty minutes, all wells were dried through prolonged vacuum aspiration (approximately 3 minutes). A developing solution was prepared immediately before use by dissolving 60 mg of 4-chloro-1-naphthal (Sigma - St. Louis, MO) in 10 ml of methanol and mixing it with 100 ml of TBS and 600 ul of 30% H_2O_2. The dotblot apparatus was then disassembled and the nitrocellulose sheet agitated in the developing solution until positive controls turned dark and known negative controls just began to darken. Development was stopped by submerging the nitrocellulose in distilled water for approximately 10 seconds and then allowing it to dry in a cool, dark area.

Dotblot controls:

All serum sample dilutions were run in duplicate wells to minimize inter-well variability. As a positive control, each dotblot sheet contained duplicate RB51-coated wells reacted with a bovine serum (Steer 66) known to have a high level of specific antibodies to RB51. Negative controls consisted of two pre-inoculation bleedings from each animal and two wells containing all appropriate reagents except serum. In addition, since high non-specific binding in wells containing no antigen had been noted in development of this protocol, 'no antigen' control wells were used for each serum sample. These wells were identical to all other wells except that: 1) only TBS was used for the antigen coating step and 2) the wells were incubated with only a 1:20 v/v serum dilution. Wells containing all reagents except conjugated antisera were not included since previous work by L. Colby indicated that the conjugated antisera did not bind nonspecifically in this system.

Dotblot readings:

Twelve hours after development, each nitrocellulose dotblot image was scanned using Adobe Photoshop LE software for the Macintosh and saved as a TIFF file. The density of each dot was determined with the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

By using a circular image selection area (0.1 sq. inch) manually superimposed over each dot, the program measured the average pixel density and standard deviation within the selection area. Each measurement was saved in a results file then imported into an Excel (Microsoft Excel version 5.0) file for data manipulation.

All dotblots were also visually evaluated. Dots from samples were compared to both prevaccination dots (negative controls) and Steer 66 dots (positive control) and then subjectively classified as positive or negative.

Antigen Preparation for Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Bacteria of interest were grown on TSA plates for 48 hours and then harvested in 10 mM Tris, pH 8.0. The bacteria were acetone killed by adding an equal volume of acetone and stirring

for 3 minutes. After washing twice in 10 mM Tris, the bacteria were resuspended in 10 mM Tris to 10% transmittance at 525 nm. This suspension was divided into 1 ml aliquots which were then centrifuged, the supernatant discarded, and the pellets resuspended in 100 ul of 10 mM Tris. This preparation was stored at - 40˚ C. Immediately before use, an aliquot was defrosted and mixed with 100 ul of Laemmli 2X sample buffer (Sigma - St. Loius, MO), boiled for five minutes, then centrifuged at 11,800 g for 3 minutes. Each gel strip was loaded with 20 ul of the resultant supernatant unless otherwise specified.

SDS - PAGE

SDS-PAGE was performed with ten channel 1.5 mm thick mini-gels as described by Laemmli (Laemmli, 1970) using 12.5% polyacrylamide slab gels. One strip of each gel was loaded with 12.5 *u*l of one broad range molecular weight standard (Novex - San Diego, CA) and 20 ul of antigen solution were loaded into each of the remaining nine strips. Electrophoresis was performed at 25 mA per gel until the dye front was within one centimeter of the bottom of the gel (approximately one hour).

Western Blotting of Elk Serum Samples

Gels were transferred onto 0.45 um pore size nitrocellulose sheets (Micron Separation Inc - Westborough, MA) at 0.14 mAmp for one hour using a semi-dry transfer apparatus (Integrated Separation Systems, MA). After removing the molecular weight marker strip, the nitrocellulose sheet was stained in 0.5% Ponceau S solution for approximately 30 seconds. The sheet was destained in distilled water until the protein bands could be readily visualized to confirm a successful transfer of proteins. The sheet was then completely destained in distilled water and the nitrocellulose sheet was agitated in TBS containing 0.25% gelatin (Difco - Detroit, MI) for one hour to block non-specific binding of antibodies. The sheet was cut into strips corresponding to the strips of transferred antigen and agitated overnight in serum diluted 1:50 (v/v) in TBS. Two serum samples (see Table 2.6) were tested for each elk: 1) serum collected on the day of vaccination and 2) the post-vaccination serum sample with the highest optical density reading in the indirect ELISA at serum sample dilutions of 1:50 (see tables 2.10 – 2.13). Serum of randomly chosen elk in each study group were examined for their reaction with RB51 whole cell antigen in Western blot to identify antigens to which elk produce antibodies. Each strip was washed five times in TBST for two minutes and then agitated with a 1:2000 dilution of monoclonal mouse anti-bovine IgG_1 (Veterinary Medical Research and Development - Pullman, WA) in TBS for one hour. After another five washings in TBST, the strips were agitated with a 1:500 dilution in TBS of horseradish peroxidase-conjugated goat anti-mouse IgG (whole molecule) (Cappel - West Chester, PA) for one hour. The strips were washed again in TBST and then twice in distilled water to remove any residual Tween which might cause non-specific color development (89). The developing solution was prepared immediately before use by mixing 60 mg of 4-chloro-1-naphthol (Sigma - St. Louis, MO) dissolved in 10 ml methanol with 100 ml of TBS and 600 ul of 30% H_2O_2. After washing, the nitrocellulose was agitated in the developing solution until positive control strips showed color development but before the entire strip turned uniformly purple. The nitrocellulose was briefly rinsed in distilled water and allowed to dry in a cool, dark area. A photograph and a digital scan was made of each strip within twelve hours of development. All photography and digital scans contained within this thesis were performed by Mr. Jerry Barber of the Reprographics Department of the Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, VA.

The centers of all visible bands were marked with a pencil directly onto the strips to locate the elk antibodies reacting with the monoclonal anti-bovine IgG_1 and the RB51 antigen. The strips were then washed five times in TBST and agitated in a 1:500 (v/v) dilution of peroxidase-conjugated rabbit anti-bovine IgG (whole molecule) (Cappel - West Chester, PA) in TBS for one hour to visualize antigens reactive with the other bovine IgG sub-isotypes. The strips were washed, developed, and dried as described above. A photograph and a digital scan was made of each strip within twelve hours of development.

TABLE 2.6. Randomly selected elk sera tested against RB51 antigen in Western blot

Elk Group:	Elk #	Serum samples tested^:
Saline inoculated	2	day 0 and 4 wks PI*
	24	day 0 and 8 wks PI
	31	day 0 and 4 wks PI
	50	day 0 and 10 wks PI
	53	day 0 and 8 wks PI
	55	day 0 and 14 wks PI
Ballistically inoculated	4	day 0 and 8 wks PI
	27	day 0 and 10 wks PI
	47	day 0 and 18 wks PI
	54	day 0 and 8 wks PI
	76	day 0 and 18 wks PI
	79	day 0 and 10 wks PI
Female SC inoculated	43	day 0 and 4 wks PI
	68	day 0 and 4 wks PI
	78	day 0 and 10 wks PI
	81	day 0 and 27 wks PI
	91	day 0 and 4 wks PI
	99	day 0 and 4 wks PI
Bull SC inoculated	6	day 0 and 14 wks PI
	32	day 0 and 10 wks PI
	36	day 0 and 8 wks PI
	84	day 0 and 8 wks PI

* PI = approximate weeks post-inoculation

^post-inoculation sera were selected based on highest OD readings in ELISA using 1:50 serum dilution.

A broad range (4 – 250 kDa) molecular weight standard run in one strip of every SDS-PAGE was used to compute the estimated molecular weight of all protein bands visualized in Western blot. The molecular weight standard was composed of the following proteins:

TABLE 2.7. Proteins and approximate molecular weights (kDa) used in Novex - MultiMark™ multi-colored standard

Protein	Approximate Molecular Weight (kDa)
myosin	250
phosphorylase B	148
glutamic dehydrogenase*	60
carbonic anhydrase*	42
myoglobin *	30
myoglobin*	22
lysozyme*	17
aprotinin	6
insulin	4

*proteins whose molecular weights can accurately be estimated with 12.5% polyacrylamide gel in SDS-PAGE

Due to the 12.5% concentration of the polyacrylamide gel used in this SDS-PAGE, bands produced only between 17 – 60 kDa protein standards could be accurately estimated. The migration distance of the 17–60 kDa proteins within the molecular weight standard were measured from the top of the gel and plotted against the $\log_{10}$ molecular weight of the protein. Linear regression (Cricket Graphics version 3.0) was used to produce a standard curve which was then used to determine the molecular weight of *Brucella* antigens reacting with the elk sera (58). All curves produced by linear regression had large r^2 values (> 0.980) indicating that the linear regression line closely approximated the original curve of $\log_{10}$ molecular weight.

Controls used in gel electrophoresis and Western blotting of elk serum samples:

After transfer, all nitrocellulose sheets were stained with 0.5% Ponceau S to show that the transfer of proteins was successful. To illustrate that proper blocking and transfer had occurred, one antigen strip of each transfer sheet was developed with a known anti-RB51 hyper-immune serum (Goat 48) with a characteristic banding pattern against RB51. Finally, testing both pre-vaccination and post-vaccination serum samples from the same elk processed on the same sheet of nitrocellulose eliminated standard experimental variability which occurs between transfers and allowed for identification of non-vaccine specific reactions.

BRU 38 Colony Blot Test

BRU 38 colony blot tests were performed as described by Roop et al. (101). This test which utilizes a monoclonal antibody to O-chain, can detect anti-O-chain antibodies in serum. Briefly, each sample to be tested was inoculated into one well of a sterile 96-well plate containing 100 ul of sterile trypticase soy broth. Two control wells each with *B. abortus* strains RB51 or 2308 were included in each plate. The plates were incubated at 37˚ C with 5% CO_2 for approximately 12 hours. A flame sterilized 48-prong replicator wand was used to transfer half of each 96-well plate onto a TSA plate. The plates were incubated at 37˚ C with 5% CO_2 for 48 hours and observed for visible growth. Cultures were then replicated on a sterile circle of 0.45 um pore size nitrocellulose

sheet by carefully placing the nitrocellulose on the TSA surface for approximately 20 seconds. The nitrocellulose was then submerged in chloroform and agitated for 20 minutes to kill all bacteria. After drying, the nitrocellulose was agitated in a blocking-digestion buffer (101) for 45 minutes, washed five times in TBST-20, and then agitated for one hour in a 1:10 dilution of the monoclonal rat antibody BRU 38 (specific for the O-side chain of smooth *Brucella spp.*) in TBS. After five washings in TBST-20, the nitrocellulose was agitated for an hour in a 1:800 v/v dilution in TBS of horseradish peroxidase conjugated polyclonal goat anti-rat IgG (Cappell - West Chester, PA). Finally, the nitrocellulose was washed five times in TBST-20 then developed in a solution of 60 mg of 4-chloro-1-naphthol dissolved in 10 ml methanol combined with 100 ml of TBS and 600 ul of 30% H_2O_2 until the positive control was clearly visible. The nitrocellulose was then briefly submerged in distilled water and allowed to dry in a cool and dry area.

RESULTS

Identification of Elk Isolates as *Brucella spp*.

Examination of each elk isolate in a variety of tests (Table 2.8) indicated that all recovered elk isolates belong to the genus *Brucella*. Concurrently tested stock cultures of *Brucella* strains RB51 and 2308 yielded the same results as the elk isolates.

TABLE 2.8. Identification of elk isolates as *Brucella spp.*

Elk Isolate	Gram stain	Lysis in 3% KOH	MacConkey agar	Christensen's urease test media	Simmon's Citrate test media	Oxidase SpotTest	Indole SpotTest	Catalase test
RB51	-	+	-	+	-	+	-	+
Biobullet RB51	-	+	-	+	-	+	-	+
Elk #20 Ampulla	-	+	-	+	-	+	-	+
Elk #20 Hemoculture	-	+	-	+	-	+	-	+
Elk #20 Prostate	-	+	-	+	-	+	-	+
Elk #20 Left Seminal Vesicle	-	+	-	+	-	+	-	+
Elk #22 Hemoculture	-	+	-	+	-	+	-	+
Elk #23 Hemoculture	-	+	-	+	-	+	-	+
Elk #36 Hemoculture	-	+	-	+	-	+	-	+
Elk #38 Spleen	-	+	-	+	-	+	-	+
Elk #62 Hemoculture	-	+	-	+	-	+	-	+
Elk #69 Hemoculture	-	+	-	+	-	+	-	+
Elk #76 Hemoculture	-	+	-	+	-	+	-	+
Brucella strain 2308	-	+	-	+	-	+	-	+

Identification of Elk Isolates as *B. abortus* strain RB51

All elk isolates were further classified as *Brucella abortus* strain RB51 using the tests listed in Table 2.9 below. The isolates behaved identically to a stock culture of RB51 and were significantly different from the stock culture of *B. abortus* strain 2308. These differences are due to the rifampicin resistance mutation in RB51 and the absence of the LPS associated O-side chain in RB51 compared to strain 2308.

TABLE 2.9. Identification of elk isolates as *B. abortus* strain RB51

Elk Isolate	Agglutination in 0.1% Acriflavin	Uptake of (0.05%) Crystal Violet Dye	Growth on 0.0125% Rifampicin plates	Growth on 0.025% Rifampicin plates	BRU 38 Colony Blot Test
RB51	+	+	+	+	-
Biobullet RB51	+	+	+	+	-
20 Ampulla	+	+	+	+	-
20 Hemoculture	+	+	+	+	-
20 Prostate	+	+	+	+	-
20 Left Seminal Vesicle	+	+	+	+	-
22 Hemoculture	+	+	+	+	-
23 Hemoculture	+	+	+	+	-
36 Hemoculture	+	+	+	+	-
38 Spleen	+	+	+	+	-
62 Hemoculture	+	+	+	+	-
69 Hemoculture	+	+	+	+	-
76 Hemoculture	+	+	+	+	-
Brucella strain 2308	-	-	-	-	+

Elk ELISA Results

ELISAs were evaluated to minimize the inter-well, inter-plate, inter-animal, and time variability inherent in all ELISAs Both 1:50 and 1:100 serum dilutions were examined for all animals. For the following data tables, many abbreviations were used to summarize the data such that all samples from each elk could be visualized on one page.

'*Group*' refers to the experimental group to which the elk belongs.

'*Dilution*' refers to the dilution of test serum used in the ELISA. *'no Ag*" rows refer to control wells not coated with RB51 antigen which were tested with all elk samples diluted 1:50 in PBST.

'*Pre 1*' and '*Pre 2*' at the 1:50 and 1:100 dilutions refer to only one of the two duplicate wells containing the indicated dilution of serum obtained two weeks prior to vaccination. *'No Ag*' '*Pre 1*' represents the **average** of two duplicate wells not coated with RB51 antigen but which were incubated with a 1:50 dilution of serum obtained two weeks prior to vaccination.

‘*Vac 1*’ and ‘*Vac 2*’ are identical to ‘*Pre 1*’ and ‘*Pre 2*’ except the serum sample used was obtained on the day of vaccination.

‘*4 wks*’, ‘*8 wks*’, ‘*10 wks*’, ‘*14 wks*’, ‘*18 wks*’, and ‘*27 wks*’ refer to the **average** of two duplicate wells at stated dilutions using serum obtained approximately 4 weeks post-inoculation (PI), 8 weeks PI, 10 weeks PI, 14 weeks PI, 18 weeks PI, and 27 weeks PI respectively.

‘*positive control*’ refers to the **average** optical density reading of two duplicate wells incubated with a 1:100 dilution of Steer 66 - a bovine serum with a known high level of specific antibodies against RB51.

‘*No serum*’ refers to the **average** optical density reading of two duplicate wells which were incubated with all reagents except test serum.

Blank cells within the table indicate that the indicated serum samples were not available for testing.

TABLE 2.10. ELISA optical densities of saline inoculated elk

Group	Elk #	2	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.039		0.052		0.043	0.039	0.036	0.044	0.040	0.042		
			1:50	0.044	0.054	0.050	0.052	0.050	0.047	0.042	0.068	0.049	0.052		
			1:100	0.043	0.043	0.062	0.059	0.086	0.047	0.045	0.054	0.054	0.054	1.578	0.055

Group	Elk #	5	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.040		0.064		0.037	0.036	0.036	0.034	0.038	0.037		
			1:50	0.039	0.039	0.040	0.058	0.044	0.038	0.042	0.038	0.040	0.050		
			1:100	0.040	0.038	0.041	0.041	0.042	0.039	0.043	0.041	0.064	0.068	1.352	0.051

Group	Elk #	8	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.036		0.040		0.039	0.038	0.036	0.034	0.059	0.048		
			1:50	0.049	0.053	0.052	0.041	0.063	0.042	0.046	0.042	0.052	0.040		
			1:100	0.051	0.046	0.119	0.042	0.050	0.107	0.044	0.041	0.040	0.042	1.378	0.044

Group	Elk #	11	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.042		0.038		0.042	0.040	0.035	0.034	0.036	0.036		
			1:50	0.056	0.039	0.059	0.062	0.042	0.041	0.044	0.041	0.038	0.041		
			1:100	0.040	0.040	0.049	0.050	0.044	0.045	0.040	0.044	0.040	0.043	1.502	0.043

Group	Elk #	24	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.042		0.038		0.038	0.041	0.035	0.040	0.037	0.046		
			1:50	0.043	0.044	0.043	0.043	0.043	0.050	0.040	0.042	0.040	0.045		
			1:100	0.042	0.042	0.044	0.042	0.045	0.048	0.044	0.040	0.040	0.045	1.544	0.049

Group	Elk #	31	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.040		0.042		0.058	0.040	0.038	0.036	0.038	0.040		
			1:50	0.070	0.045	0.051	0.048	0.057	0.050	0.046	0.042	0.046	0.050		
			1:100	0.049	0.047	0.048	0.045	0.048	0.048	0.046	0.044	0.054	0.052	1.424	0.045

note: all values at 4 wks, 8 wks, 10 wks, 14 wks, 18 wks and 27 wks represent the average of two identical wells.

TABLE 2.10. ELISA optical densities of saline inoculated elk (cont'd)

Group	Elk #	33	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.040		0.052		0.037	0.038	0.035	0.035	0.036	0.036		
			1:50	0.043	0.041	0.043	0.045	0.044	0.042	0.042	0.043	0.043	0.045		
			1:100	0.045	0.045	0.073	0.066	0.044	0.064	0.062	0.048	0.059	0.050	1.448	0.048

Group	Elk #	38	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.034		0.034	0.057	0.036							
			1:50	0.040	0.039	0.039	0.039	0.044							
			1:100	0.040	0.041	0.039	0.039	0.040						1.468	0.046

Group	Elk #	50	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.072		0.038		0.040	0.040	0.038	0.052	0.044	0.045		
			1:50	0.046	0.067	0.048	0.042	0.046	0.051	0.061	0.046	0.042	0.046		
			1:100	0.047	0.048	0.049	0.048	0.047	0.048	0.046	0.046	0.048	0.048	1.425	0.062

Group	Elk #	53	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.036		0.035		0.038	0.036	0.034	0.034	0.037	0.038		
			1:50	0.039	0.040	0.040	0.040	0.040	0.043	0.040	0.040	0.038	0.040		
			1:100	0.039	0.039	0.042	0.040	0.041	0.044	0.040	0.042	0.048	0.050	1.544	0.050

Group	Elk #	55	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.340		0.036		0.036	0.036	0.036	0.035	0.034	0.035		
			1:50	0.046	0.042	0.078	0.042	0.043	0.044	0.045	0.043	0.040	0.043		
			1:100	0.043	0.045	0.044	0.042	0.040	0.042	0.043	0.048	0.042	0.043	1.474	0.045

Group	Elk #	58	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.036		0.036		0.044	0.040	0.036	0.036	0.037	0.036		
			1:50	0.093	0.116	0.058	0.053	0.048	0.049	0.044	0.043	0.044	0.042		
			1:100	0.068	0.073	0.046	0.045	0.048	0.046	0.046	0.044	0.044	0.046	1.638	0.054

note: all values at 4 wks, 8 wks, 10 wks, 14 wks, 18 wks and 27 wks represent the average of two identical wells.

TABLE 2.10. ELISA optical densities of saline inoculated elk (cont'd)

Group	Elk #	63	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.036		0.036		0.037	0.037	0.034	0.034	0.037			
			1:50	0.045	0.042	0.043	0.043	0.045	0.042	0.043	0.046	0.047			
			1:100	0.045	0.042	0.045	0.043	0.044	0.044	0.044	0.030	0.048		1.638	0.054

Group	Elk #	66	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.034		0.035		0.037	0.036	0.033	0.034	0.034	0.033		
			1:50	0.038	0.040	0.041	0.038	0.040	0.042	0.040	0.040	0.036	0.040		
			1:100	0.038	0.038	0.039	0.039	0.038	0.038	0.038	0.039	0.038	0.040	1.356	0.042

Group	Elk #	74	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.050		0.047		0.049	0.050	0.046	0.046	0.048	0.048		
			1:50	0.047	0.046	0.048	0.048	0.050	0.050	0.045	0.046	0.046	0.048		
			1:100	0.037	0.036	0.035	0.037	0.040	0.036	0.036	0.038	0.039	0.040	1.486	0.048

Group	Elk #	82	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Saline			no Ag	0.038		0.040		0.038	0.040	0.038	0.038	0.039	0.038		
			1:50	0.050	0.050	0.051	0.052	0.051	0.050	0.050	0.049	0.052	0.048		
			1:100	0.052	0.054	0.050	0.051	0.050	0.048	0.047	0.051	0.052	0.051	1.739	0.050

note: all values at 4 wks, 8 wks, 10 wks, 14 wks, 18 wks and 27 wks represent the average of two identical wells.

TABLE 2.10. ELISA optical densities of saline inoculated elk (cont'd)

Group	Elk #	4	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.074		0.082		0.043	0.038	0.034	0.033	0.035	0.034		
			1:50	0.041	0.062	0.055	0.060	0.087	0.121	0.120	0.056	0.043	0.036		
			1:100	0.039	0.037	0.039	0.035	0.056	0.165	0.076	0.050	0.042	0.044	1.028	0.042

Group	Elk #	25	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.038		0.040		0.044	0.038	0.034	0.033	0.034	0.034		
			1:50	0.040	0.037	0.037	0.038	0.072	0.114	0.093	0.102	0.096	0.156		
			1:100	0.040	0.041	0.039	0.039	0.124	0.122	0.060	0.071	0.060	0.073	1.061	0.039

Group	Elk #	27	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.034		0.038		0.034	0.036	0.037	0.034	0.034	0.034		
			1:50	0.039	0.038	0.037	0.038	0.128	0.184	0.233	0.162	0.124	0.133		
			1:100	0.045	0.046	0.063	0.039	0.158	0.218	0.221	0.131	0.108	0.086	1.382	0.038

Group	Elk #	30	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.034		0.036		0.035	0.036	0.033	0.033	0.034	0.033		
			1:50	0.037	0.036	0.037	0.038	0.060	0.147	0.160	0.120	0.089	0.044		
			1:100	0.038	0.036	0.038	0.039	0.060	0.174	0.086	0.068	0.052	0.040	1.244	0.036

Group	Elk #	37	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.044		0.040		0.038	0.044	0.044	0.040	0.040	0.040		
			1:50	0.048	0.047	0.047	0.049	0.302	0.658	0.647	0.388	0.180	0.079		
			1:100	0.046	0.048	0.050	0.051	0.214	0.616	0.586	0.256	0.111	0.062	1.406	0.048

Group	Elk #	40	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.046		0.041		0.042	0.044	0.044	0.038	0.044	0.040		
			1:50	0.056	0.055	0.047	0.051	0.120	0.247	0.200	0.184	0.240	0.070		
			1:100	0.051	0.048	0.050	0.047	0.080	0.218	0.164	0.140	0.135	0.053	1.315	0.058

note: all values at 4 wks, 8 wks, 10 wks, 14 wks, 18 wks and 27 wks represent the average of two identical wells.

TABLE 2.11. ELISA optical densities of ballistically inoculated elk

Group	Elk #	41	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.039		0.037		0.038	0.048	0.053	0.049	0.044	0.038		
			1:50	0.054	0.047	0.047	0.049	0.136	0.404	0.228	0.310	0.360	0.160		
			1:100	0.061	0.047	0.049	0.047	0.098	0.442	0.228	0.240	0.252	0.088	1.364	0.092

Group	Elk #	46	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.042		0.040		0.040	0.040	0.037	0.037	0.040	0.038		
			1:50	0.049	0.048	0.046	0.049	0.234	0.304	0.148	0.184	0.340	0.306		
			1:100	0.044	0.045	0.055	0.046	0.278	0.374	0.160	0.176	0.288	0.271	1.292	0.044

Group	Elk #	47	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.039		0.106		0.038	0.040	0.036	0.034	0.036	0.036		
			1:50	0.048	0.048	0.046	0.044	0.092	0.246	0.189	0.184	0.338	0.145		
			1:100	0.046	0.043	0.045	0.044	0.076	0.222	0.218	0.170	0.288	0.076	1.629	0.057

Group	Elk #	49	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.037		0.037		0.038	0.040	0.037	0.036	0.039	0.038		
			1:50	0.047	0.053	0.051	0.049	0.100	0.238	0.202	0.124	0.140	0.116		
			1:100	0.045	0.045	0.051	0.047	0.069	0.270	0.218	0.080	0.092	0.068	1.660	0.062

Group	Elk #	54	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.054		0.066		0.062	0.044	0.038	0.034	0.038	0.038		
			1:50	0.044	0.045	0.043	0.043	0.138	0.307	0.186	0.134	0.212	0.158		
			1:100	0.049	0.047	0.043	0.043	0.098	0.265	0.186	0.114	0.178	0.083	1.665	0.048

Group	Elk #	59	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.060		0.048		0.039	0.044	0.054	0.045	0.041	0.038		
			1:50	0.056	0.047	0.048	0.049	0.136	0.240	0.258	0.236	0.175	0.098		
			1:100	0.056	0.050	0.051	0.050	0.096	0.285	0.284	0.210	0.140	0.064	1.740	0.050

note: all values at 4 wks, 8 wks, 10 wks, 14 wks, 18 wks and 27 wks represent the average of two identical wells.

TABLE 2.11. ELISA optical densities of ballistically inoculated elk (cont'd)

Group	Elk #	67	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.043		0.044		0.040	0.038	0.040	0.036	0.037	0.036		
			1:50	0.045	0.047	0.047	0.046	0.090	0.291	0.238	0.230	0.315	0.249		
			1:100	0.047	0.045	0.045	0.042	0.070	0.373	0.290	0.280	0.358	0.161	1.590	0.052

Group	Elk #	69	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.049		0.040		0.040	0.039	0.038	0.040	0.038	0.036		
			1:50	0.047	0.120	0.046	0.050	0.114	0.310	0.344	0.139	0.076	0.046		
			1:100	0.053	0.048	0.051	0.047	0.115	0.342	0.308	0.085	0.054	0.048	1.650	0.044

Group	Elk #	70	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.040		0.040		0.042	0.038	0.040	0.037	0.037	0.036		
			1:50	0.051	0.048	0.049	0.047	0.182	0.402	0.352	0.243	0.084	0.047		
			1:100	0.055	0.055	0.053	0.053	0.180	0.396	0.365	0.124	0.069	0.052	1.772	0.052

Group	Elk #	72	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.056		0.040		0.040	0.052	0.046	0.044	0.041	0.038		
			1:50	0.047	0.046	0.049	0.050	0.161	0.376	0.238	0.222	0.242	0.080		
			1:100	0.045	0.046	0.056	0.046	0.158	0.420	0.284	0.231	0.154	0.062	1.798	0.048

Group	Elk #	76	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.038		0.036		0.043	0.040	0.044	0.046	0.038	0.040		
			1:50	0.054	0.045	0.046	0.050	0.198	0.336	0.314	0.300	0.346	0.318		
			1:100	0.057	0.049	0.048	0.049	0.159	0.353	0.302	0.269	0.239	0.200	1.721	0.074

Group	Elk #	79	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
Ballistic			no Ag	0.043		0.059		0.047	0.054	0.044	0.036	0.038	0.037		
			1:50	0.056	0.055	0.053	0.058	0.162	0.331	0.688	0.533	0.231	0.074		
			1:100	0.057	0.058	0.056	0.057	0.124	0.390	0.496	0.404	0.138	0.062	1.692	0.056

note: all values at 4 wks, 8 wks, 10 wks, 14 wks, 18 wks and 27 wks represent the average of two identical wells.

TABLE 2.12. ELISA optical densities of subcutaneously inoculated female elk

Group	Elk # 7	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.063		0.040		0.051	0.036	0.054	0.034	0.036	0.036		
		1:50	0.045	0.090	0.066	0.043	0.249	0.109	0.086	0.048	0.060	0.050		
		1:100	0.040	0.042	0.045	0.044	0.276	0.075	0.068	0.044	0.052	0.046	1.336	0.062

Group	Elk # 9	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.040		0.040		0.040	0.038	0.051	0.038	0.037	0.039		
		1:50	0.047	0.041	0.042	0.042	0.250	0.234	0.213	0.113	0.134	0.053		
		1:100	0.043	0.042	0.047	0.048	0.654	0.276	0.142	0.064	0.066	0.047	1.427	0.046

Group	Elk # 39	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.037		0.036		0.037	0.037	0.061	0.034	0.038	0.036		
		1:50	0.042	0.042	0.043	0.045	0.404	0.296	0.197	0.069	0.070	0.048		
		1:100	0.052	0.044	0.045	0.047	0.479	0.299	0.151	0.055	0.052	0.048	1.285	0.144

Group	Elk # 43	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.035		0.038		0.036	0.037	0.034	0.035	0.037	0.037		
		1:50	0.042	0.040	0.045	0.043	0.260	0.241	0.142	0.090	0.097	0.046		
		1:100	0.041	0.042	0.043	0.043	0.302	0.200	0.084	0.064	0.069	0.048	1.351	0.084

Group	Elk # 45	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.035		0.036		0.032		0.033	0.034	0.036	0.036		
		1:50	0.038	0.038	0.039	0.040	0.367		0.277	0.236	0.181	0.131		
		1:100	0.040	0.041	0.038	0.041	0.318		0.121	0.071	0.064	0.055	1.356	0.042

Group	Elk # 48	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.039		0.038		0.037	0.037	0.039	0.035	0.036	0.036		
		1:50	0.049	0.066	0.045	0.050	0.338	0.264	0.280	0.186	0.052	0.046		
		1:100	0.050	0.056	0.046	0.047	0.367	0.198	0.182	0.104	0.045	0.048	1.302	0.048

note: all values at 4 wks, 8 wks, 10 wks, 14 wks, 18 wks and 27 wks represent the average of two identical wells.

TABLE 2.12. ELISA optical densities of subcutaneously inoculated female elk (cont'd)

Group	Elk # 56	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.039		0.038		0.046	0.060	0.040	0.036	0.036	0.036		
		1:50	0.048	0.046	0.045	0.045	0.631	0.446	0.365	0.204	0.172	0.088		
		1:100	0.048	0.045	0.044	0.045	0.550	0.377	0.246	0.138	0.088	0.060	1.580	0.050

Group	Elk # 57	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.038		0.037		0.039	0.038	0.036	0.036	0.038	0.040		
		1:50	0.046	0.044	0.047	0.049	0.887	0.306	0.226	0.217	0.216	0.193		
		1:100	0.048	0.114	0.048	0.048	0.670	0.278	0.145	0.167	0.160	0.110	1.440	0.048

Group	Elk # 60	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.038		0.037		0.040	0.040	0.038	0.035	0.038	0.036		
		1:50	0.082	0.049	0.049	0.048	0.400	0.371	0.302	0.300	0.350	0.197		
		1:100	0.115	0.051	0.049	0.044	0.332	0.302	0.228	0.236	0.289	0.086	1.473	0.050

Group	Elk # 61	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.036		0.034		0.053	0.038	0.038	0.040	0.042			
		1:50	0.040	0.039	0.041	0.042	0.664	0.112	0.043	0.045	0.044			
		1:100	0.039	0.038	0.041	0.042	0.576	0.044	0.041	0.040	0.044		1.468	0.046

Group	Elk # 62	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.036		0.035		0.035	0.034	0.036	0.036	0.036	0.035		
		1:50	0.051	0.046	0.051	0.051	0.380	0.322	0.316	0.372	0.404	0.258		
		1:100	0.060	0.048	0.056	0.049	0.360	0.301	0.282	0.282	0.332	0.153	1.576	0.050

Group	Elk # 68	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.039		0.035		0.036	0.037	0.036	0.035	0.034	0.036		
		1:50	0.060	0.045	0.048	0.045	0.356	0.348	0.304	0.278	0.280	0.252		
		1:100	0.327	0.047	0.143	0.045	0.380	0.318	0.306	0.251	0.154	0.131	1.553	0.048

note: all values at 4 wks, 8 wks, 10 wks, 14 wks, 18 wks and 27 wks represent the average of two identical wells.

TABLE 2.12. ELISA optical densities of subcutaneously inoculated female elk (cont'd)

Group	Elk # 71	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.034		0.036		0.036	0.034	0.033	0.033	0.042	0.032		
		1:50	0.046	0.040	0.046	0.044	0.326	0.354	0.278	0.330	0.472	0.378		
		1:100	0.046	0.040	0.041	0.041	0.322	0.354	0.265	0.303	0.418	0.236	1.500	0.044

Group	Elk # 75	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.038		0.036		0.038	0.036	0.036	0.033	0.044	0.033		
		1:50	0.045	0.043	0.044	0.043	0.435	0.315	0.338	0.409	0.431	0.418		
		1:100	0.043	0.042	0.043	0.042	0.444	0.322	0.364	0.380	0.415	0.263	1.488	0.048

Group	Elk # 77	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.047		0.042		0.046	0.041	0.054	0.035	0.038	0.039		
		1:50	0.052	0.062	0.046	0.046	0.272	0.337	0.380	0.416	0.304	0.332		
		1:100	0.052	0.047	0.045	0.046	0.305	0.394	0.394	0.360	0.289	0.262	1.528	0.050

Group	Elk # 78	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.040		0.037		0.037	0.038	0.037	0.034	0.036	0.036		
		1:50	0.048	0.048	0.048	0.047	0.325	0.340	0.354	0.299	0.130	0.067		
		1:100	0.044	0.051	0.046	0.043	0.422	0.420	0.316	0.200	0.074	0.056	1.678	0.048

Group	Elk # 81	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.038		0.036		0.048	0.038	0.041	0.038	0.037	0.035		
		1:50	0.048	0.048	0.050	0.051	0.306	0.176	0.256	0.300	0.372	0.374		
		1:100	0.047	0.049	0.048	0.052	0.374	0.162	0.166	0.228	0.262	0.271	1.565	0.066

Group	Elk # 89	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.042		0.048		0.046	0.040	0.050	0.038	0.038	0.036		
		1:50	0.049	0.052	0.047	0.054	0.378	0.227	0.300	0.168	0.156	0.097		
		1:100	0.050	0.047	0.049	0.052	0.334	0.137	0.138	0.077	0.072	0.062	1.606	0.058

note: all values at 4 wks, 8 wks, 10 wks, 14 wks, 18 wks and 27 wks represent the average of two identical wells.

TABLE 2.12. ELISA optical densities of subcutaneously inoculated female elk (cont'd)

Group	Elk # 91	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.038		0.038		0.040	0.038	0.046	0.035	0.038	0.039		
		1:50	0.050	0.054	0.049	0.049	0.200	0.124	0.068	0.055	0.052	0.160		
		1:100	0.054	0.053	0.046	0.049	0.168	0.078	0.060	0.052	0.051	0.106	1.508	0.051

Group	Elk # 99	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	27 wks	+ control	no serum
SC Female		no Ag	0.036		0.036		0.036	0.035	0.036	0.036	0.036	0.036		
		1:50	0.050	0.048	0.047	0.047	0.342	0.329	0.247	0.196	0.254	0.098		
		1:100	0.050	0.047	0.045	0.046	0.383	0.278	0.201	0.184	0.186	0.066	1.495	0.048

note: all values at 4 wks, 8 wks, 10 wks, 14 wks, 18 wks and 27 wks represent the average of two identical wells.

TABLE 2.13. ELISA optical densities of subcutaneously inoculated bull elk

Group	Elk #	3	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	+ control	no serum
SC Bull			no Ag	0.038		0.046		0.051	0.048	0.040	0.044	0.041		
			1:50	0.041	0.046	0.050	0.049	0.274	0.242	0.124	0.184	0.073		
			1:100	0.057	0.048	0.050	0.049	0.257	0.152	0.082	0.092	0.059	1.578	0.055

Group	Elk #	6	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	+ control	no serum
SC Bull			no Ag	0.038		0.036		0.040	0.046	0.038	0.036	0.040		
			1:50	0.043	0.046	0.041	0.044	0.284	0.242	0.249	0.292	0.206		
			1:100	0.051	0.048	0.043	0.043	0.286	0.162	0.182	0.260	0.184	1.352	0.051

Group	Elk #	10	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	+ control	no serum
SC Bull			no Ag	0.048		0.036		0.044	0.034	0.041	0.036	0.038		
			1:50	0.058	0.040	0.047	0.041	0.286	0.156	0.163	0.090	0.082		
			1:100	0.051	0.045	0.040	0.041	0.318	0.099	0.086	0.055	0.054	1.378	0.044

Group	Elk #	20	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	+ control	no serum
SC Bull			no Ag	0.040		0.034		0.046	0.039	0.034	0.034	0.038		
			1:50	0.039	0.051	0.041	0.039	0.405	0.358	0.416	0.342	0.336		
			1:100	0.039	0.039	0.038	0.038	0.400	0.324	0.221	0.148	0.158	1.502	0.043

Group	Elk #	22	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	+ control	no serum
SC Bull			no Ag	0.037		0.036		0.036	0.036	0.046	0.036	0.041		
			1:50	0.041	0.042	0.049	0.042	0.374	0.441	0.384	0.379	0.046		
			1:100	0.053	0.057	0.043	0.041	0.252	0.465	0.289	0.289	0.050	1.544	0.049

note: all values at 4 wks, 8 wks, 10 wks, 14 wks, 18 wks and 27 wks represent the average of two identical wells.

TABLE 2.13. ELISA optical densities of subcutaneously inoculated bull elk (cont'd)

Group	Elk #	23	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	+ control	no serum
SC Bull			no Ag	0.039		0.037		0.058	0.052	0.046	0.048	0.038		
			1:50	0.051	0.049	0.117	.0.062	0.292	0.416	0.220	0.308	0.239		
			1:100	0.051	0.047	0.046	0.047	0.357	0.426	0.209	0.204	0.098	1.424	0.045

Group	Elk #	32	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	+ control	no serum
SC Bull			no Ag	0.038		0.037		0.036	0.036	0.036	0.042	0.038		
			1:50	0.052	0.045	0.044	0.044	0.319	0.556	0.736	0.357	0.458		
			1:100	0.054	0.046	0.043	0.044	0.401	0.572	0.565	0.172	0.262	1.448	0.048

Group	Elk #	36	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	+ control	no serum
SC Bull			no Ag	0.040		0.040		0.040	0.049	0.038	0.037	0.037		
			1:50	0.047	0.050	0.047	0.047	0.308	0.366	0.345	0.348	0.300		
			1:100	0.049	0.050	0.046	0.045	0.355	0.354	0.309	0.201	0.215	1.425	0.062

Group	Elk #	84	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	+ control	no serum
SC Bull			no Ag	0.033		0.036		0.034	0.038	0.033	0.034	0.036		
			1:50	0.042	0.038	0.039	0.042	0.318	0.452	0.354	0.272	0.142		
			1:100	0.044	0.041	0.039	0.039	0.462	0.334	0.235	0.150	0.068	1.544	0.050

Group	Elk #	85	Dilution:	Pre 1	Pre 2	Vac 1	Vac 2	4 wks	8 wks	10 wks	14 wks	18 wks	+ control	no serum
SC Bull			no Ag	0.036		0.034		0.036	0.035	0.036	0.032	0.034		
			1:50	0.044	0.043	0.042	0.045	0.224	0.179	0.246	0.199	0.195		
			1:100	0.042	0.039	0.038	0.039	0.268	0.262	0.194	0.156	0.138	1.474	0.045

note: all values at 4 wks, 8 wks, 10 wks, 14 wks, 18 wks and 27 wks represent the average of two identical wells

.

ELISAs were evaluated to minimize the inter-well, inter-plate, inter-animal, and time variability inherent in all ELISAs. Both 1:50 and 1:100 serum dilutions were examined for all animals.

A percent positivity value was calculated for all elk samples. Percent positivity is designed to represent where a sample lies along a straight continuum with no binding of antibody being equivalent to 0% positivity and the antibody binding of the positive control being equivalent to 100% positivity. Therefore, the percent positivity of each sample represents the ratio of specific antibody in that sample to the amount of specific antibody in the positive control.

The % positivity of each sample was calculated with the following equation which was devised with the help of a statistician:

$$\% \text{ positivity} = 100 \times \frac{\text{(test sample O.D.) - (pre-inoculation sera O.D.)}}{\text{(positive control sera O.D.) - (no serum sample O.D.)}}$$

where:

specific O.D. of test sera =

(O.D. of elk test sample) - (average O.D. of all pre-inoculation samples* from that elk).

* (Pre 1, Pre 2, Vac 1, Vac 2)

Non-specific binding for each animal sera was partially corrected for by calculating the specific optical density for each animal's sample. Since no 'prevaccination' sample was available for the positive control sample (Steer 66), non-specific binding of the sera was approximated by subtracting the O.D. of a well that contained all reagents except serum from the O.D. of the positive control.

A negative cutoff value was determined by computing the average plus three standard deviations of the percent positivity values of all saline elk (4 weeks PI through 27 weeks post-inoculation) as described in the following equation:

negative cutoff value =

average percent positivity value of all saline inoculated samples

+

3 (SD of the percent positivity of all saline inoculated samples)

Any serum sample with a percent positivity value below this cut-off was considered to not have a significant antibody response in this test.

Upon examining the 1:100 serum dilutions, all but one (Elk # 8, 8 Wks PI, % positivity = 3.1) saline inoculated elk sample fell below that dilution's calculated negative cut-off value of 2.5% positivity. To accommodate this sample, a suspect range from 2.51-3.19% positivity was

established. The upper-most value of the suspect range was chosen to include the outlying saline elk's value. Creation of the suspect range's maximum value cannot be supported statistically and thus can only be arbitrarily assigned. Therefore, all samples with a percent positivity value > 3.20% positivity were considered to be positive.

TABLE 2.14. Classification of ELISA percent positivity values for elk sera diluted 1:100.

Classification of serum samples	Range of percent positivity
negative	0–2.50%
suspect	2.51–3.19%
positive	> 3.19%

The percent positivity of each sample diluted 1:100 in the ELISA is listed below in tables 2.15 to 2.18. Classification of values as positive, suspicious, or negative can be identified by cell shading. Positive values (> 3.19 percent positivity) are shaded with a dark gray background while suspect values (2.51 - 3.19 percent positivity) are shaded with a light gray pattern. Negative values (< 2.51 percent positivity) have no shading.

TABLE 2.15. Percent positivity of saline inoculated elk as determined by ELISA (serum dilution 1:100)

Elk #	4 Wks PI	8 Wks PI	10 Wks PI	14 Wks PI	18 Wks PI	27 Wks PI
2	2.25	-0.31	-0.44	0.15	0.15	0.15
5	0.15	-0.08	0.23	0.08	1.84	2.15
8	-1.09	3.19	-1.54	-1.76	-1.84	-1.69
11	-0.05	0.02	-0.33	-0.05	-0.33	-0.12
24	0.17	0.37	0.10	-0.17	-0.17	0.17
31	0.05	0.05	-0.09	-0.24	0.49	0.34
33	-0.95	0.48	0.34	-0.66	0.13	-0.52
38	0.02	-2.80	-2.80	-2.80	-2.80	-2.80
50	-0.07	0.00	-0.15	-0.15	0.00	0.00
53	0.07	0.27	0.00	0.13	0.54	0.67
55	-0.24	-0.10	-0.03	0.31	-0.10	-0.03
58	-0.63	-0.76	-0.76	-0.88	-0.88	-0.76
63	0.02	0.02	0.02	-0.87	0.27	-2.76
66	-0.04	-0.04	-0.04	0.04	-0.04	0.11
74	0.26	-0.02	-0.02	0.12	0.19	0.26
82	-0.10	-0.22	-0.28	-0.04	0.01	-0.04

*approximate weeks post-inoculation
shaded cells represent samples classified as positive based on a negative cutoff value of 2.50%
cross-hatched cells represent samples classified as suspect based on a suspect range of 2.51–3.19% positivity.

TABLE 2.16. Percent positivity of ballistically inoculated elk as determined by ELISA (serum dilution 1:100)

Elk #	4 Wks PI	8 Wks PI	10 Wks PI	14 Wks PI	18 Wks PI	27 Wks PI
4	1.88	12.93	3.90	1.27	0.46	0.66
25	8.24	8.05	1.98	3.06	1.98	3.25
27	8.17	12.63	12.85	6.16	4.45	2.81
30	1.84	11.28	3.99	2.50	1.18	0.19
37	12.17	41.77	39.56	15.26	4.58	0.98
40	2.47	13.44	9.15	7.24	6.84	0.32
41	3.69	30.74	13.92	14.86	15.80	2.91
46	18.47	26.16	9.01	10.30	19.27	17.91
47	2.00	11.29	11.04	7.98	15.49	2.00
49	1.38	13.95	10.70	2.07	2.82	1.31
54	3.25	13.57	8.69	4.24	8.19	2.32
59	2.62	13.80	13.74	9.36	5.22	0.72
67	1.64	21.34	15.95	15.30	20.37	7.56
69	4.06	18.20	16.08	2.19	0.26	-0.11
70	7.33	19.88	18.08	4.07	0.87	-0.12
72	6.27	21.24	13.47	10.44	6.04	0.79
76	6.57	18.35	15.26	13.25	11.43	9.06
79	4.10	20.35	26.83	21.21	4.95	0.31

*approximate weeks post-inoculation
shaded cells represent samples classified as positive based on a negative cutoff value of 2.50%
cross-hatched cells represent samples classified as suspect based on a suspect range of 2.51–3.19% positivity

Table 2.17. Percent positivity of subcutaneously inoculated female elk as determined by ELISA (serum dilution 1:100)

Elk #	4 Wks PI	8 Wks PI	10 Wks PI	14 Wks PI	18 Wks PI	27 Wks PI
7	18.29	2.51	1.96	0.08	0.71	0.24
9	44.1	16.73	7.02	1.38	1.52	0.14
39	37.86	22.09	9.11	0.7	0.44	0.09
43	20.52	12.47	3.31	1.74	2.13	0.47
45	21.16	-3.04	6.16	2.36	1.83	1.14
48	25.28	11.8	10.53	4.31	-0.4	-0.16
56	32.94	21.63	13.07	6.01	2.75	0.92
57	43.46	15.3	5.75	7.33	6.82	3.23
60	18.76	16.65	11.45	12.02	15.74	1.48
61	37.69	0.28	0.07	0	0.28	-2.81
62	20.12	16.25	15.01	15.01	18.28	6.55
68	15.88	11.76	10.96	7.31	0.86	-0.66
71	19.23	21.43	15.32	17.93	25.82	13.32
75	27.85	19.38	22.29	23.4	25.83	15.28
77	17.39	23.41	23.41	21.11	16.31	14.48
78	23.07	22.94	16.56	9.45	1.72	0.61
81	21.68	7.54	7.81	11.94	14.21	14.81
89	18.35	5.62	5.68	1.74	1.42	0.78
91	8.03	1.85	0.62	0.07	0	3.77
99	23.22	15.96	10.64	9.47	9.61	1.31

*approximate weeks post-inoculation
shaded cells represent samples classified as positive based on a negative cutoff value of 2.50%
cross-hatched cells represent samples classified as suspect based on a suspect range of 2.51–3.19% positivity.

TABLE 2.18. Percent positivity of subcutaneously inoculated bull elk as determined by ELISA (serum dilution 1:100)

Elk #	4 Wks PI	8 Wks PI	10 Wks PI	14 Wks PI	18 Wks PI
3	13.55	6.64	2.04	2.7	0.53
6	18.46	8.92	10.46	16.46	10.62
10	20.6	4.14	3.16	0.83	0.75
20	24.73	19.52	12.47	7.47	8.15
22	13.53	27.73	16	16	0.07
23	22.39	27.39	11.67	11.3	3.62
32	25.29	37.5	37	8.93	15.36
36	22.57	22.5	19.19	11.25	12.28
84	28.26	19.66	13.02	7.32	1.81
85	15.94	15.52	10.77	8.11	6.85

*approximate weeks post-inoculation

shaded cells represent samples classified as positive based on a negative cutoff value of 2.50%

cross-hatched cells represent samples classified as suspect based on a suspect range of 2.51–3.19% positivity.

Figures 2.1 - 2.5 are scatter graphs of the percent positivity of each elk's serum sample within their respective groups. ELISAs were performed with whole cell RB51 antigen, elk sera diluted 1:100, mouse monoclonal anti-bovine IgG_1 and goat anti-mouse IgG (whole molecule). Percent positivity was calculated as: percent positivity =100 X [specific O.D. of test sera / ((positive control O.D.) - (no serum control O.D.))]. Note that figure 2.1 (saline inoculated elk) has a smaller y-axis scale. The top line at 3.1% positivity represents the upper-most limit of the suspect range. The lower dashed line at 2.5 % positivity represents the negative cutoff value in this system. The numbers in the box on the right of each figure represent the individual elk identification numbers.

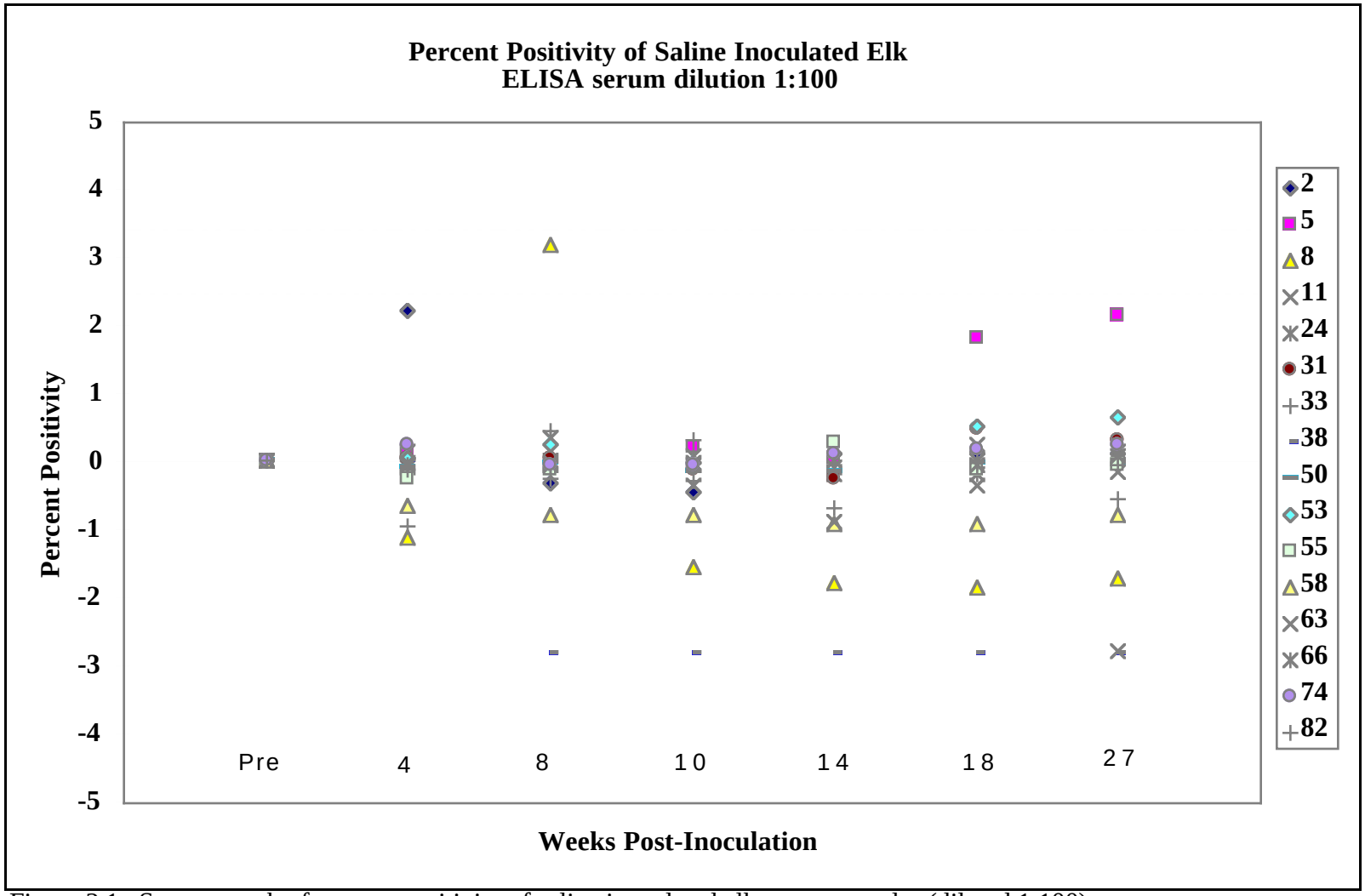

Figure 2.1. Scatter graph of percent positivity of saline inoculated elk serum samples (diluted 1:100)

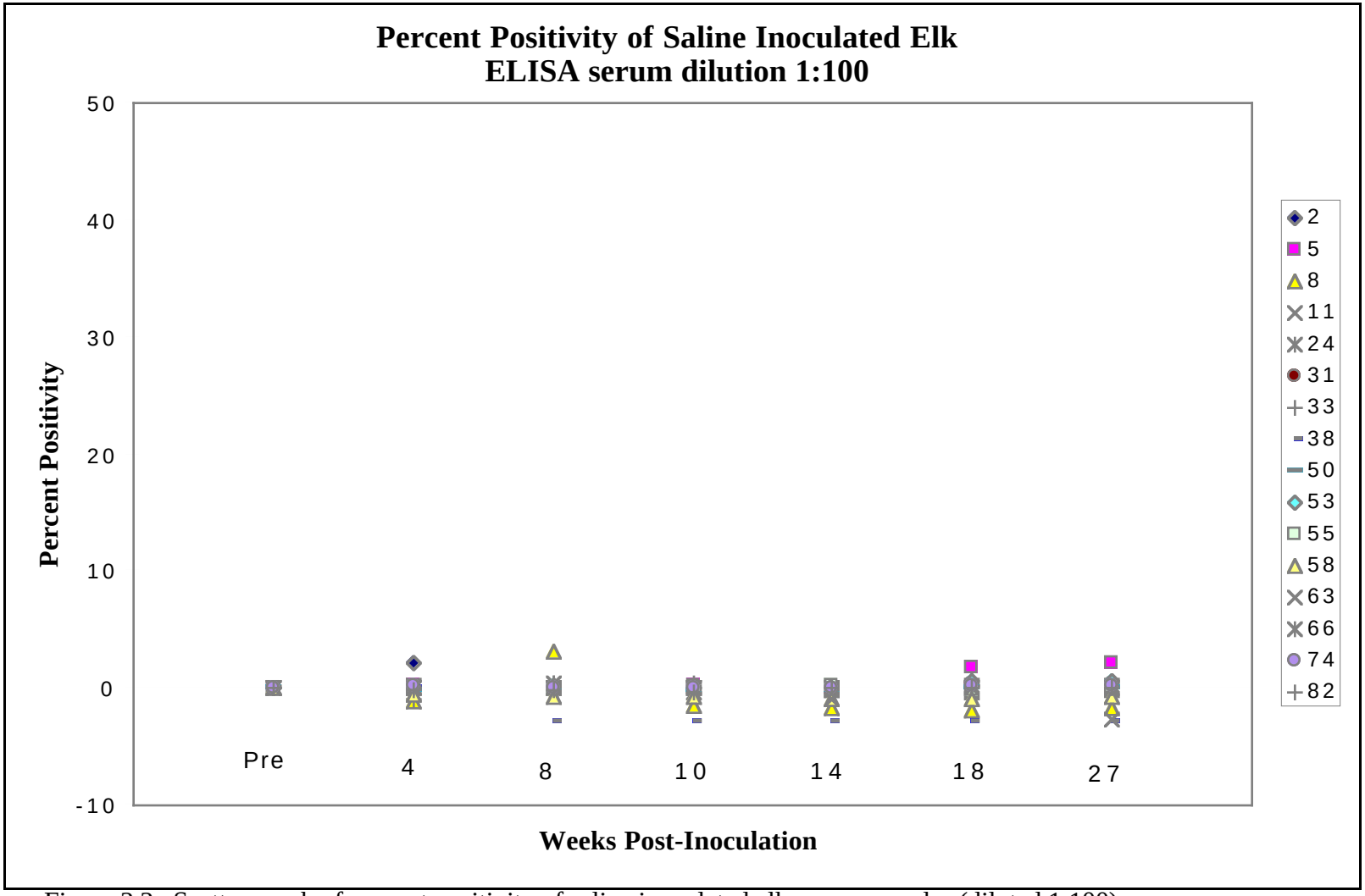

Figure 2.2. Scatter graph of percent positivity of saline inoculated elk serum samples (diluted 1:100)

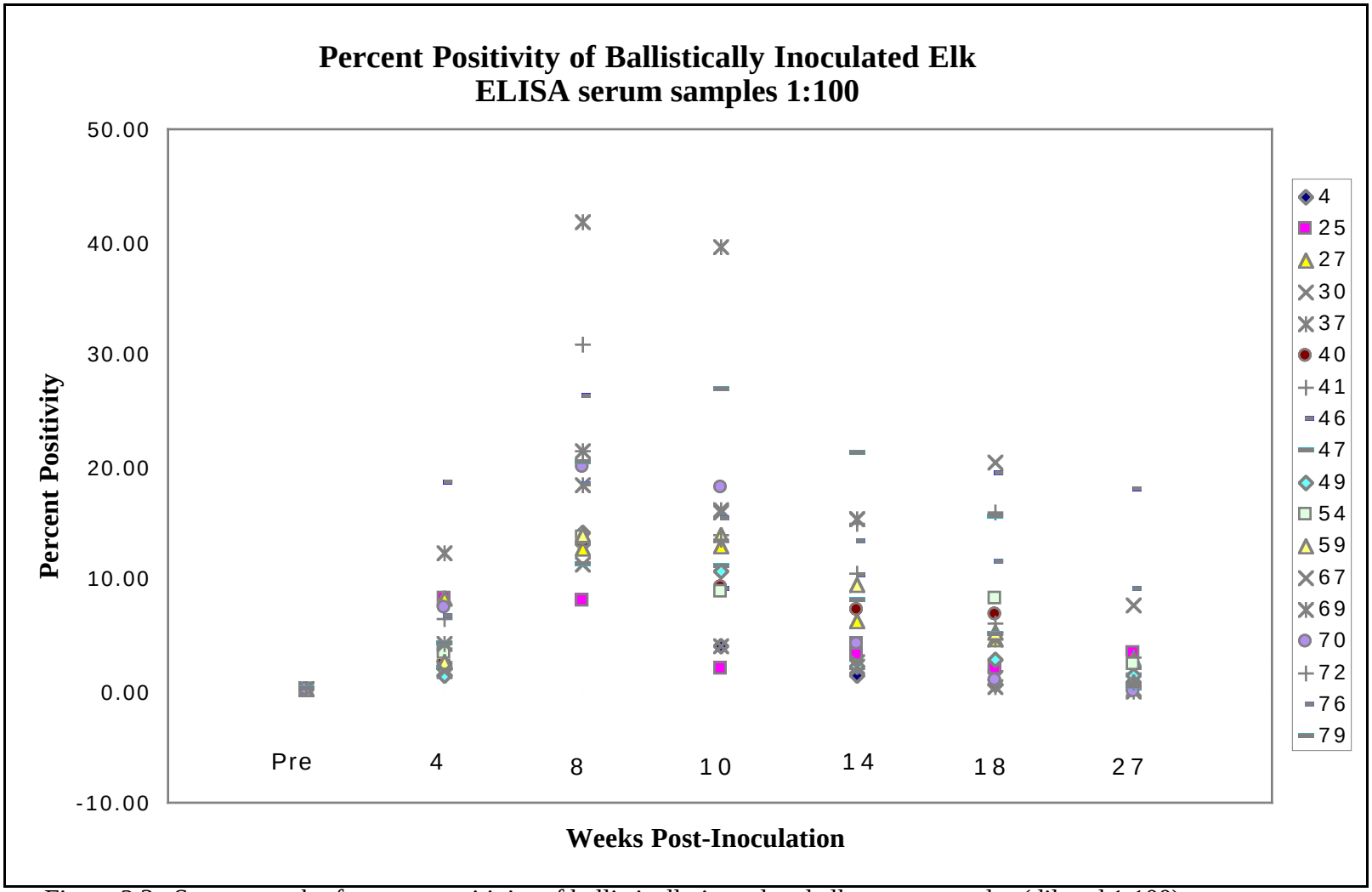

Figure 2.3. Scatter graph of percent positivity of ballistically inoculated elk serum samples (diluted 1:100).

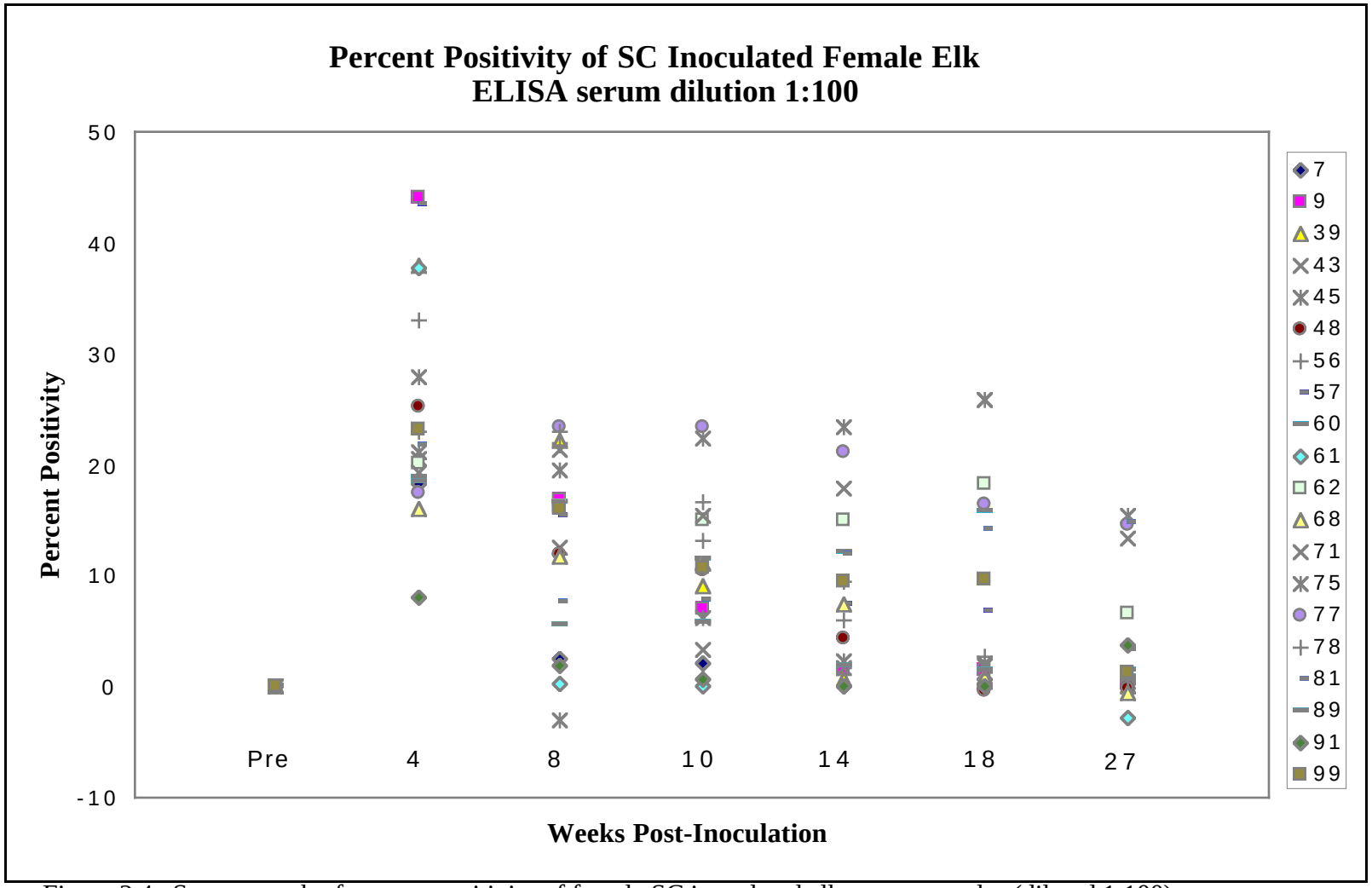

Figure 2.4. Scatter graph of percent positivity of female SC inoculated elk serum samples (diluted 1:100).

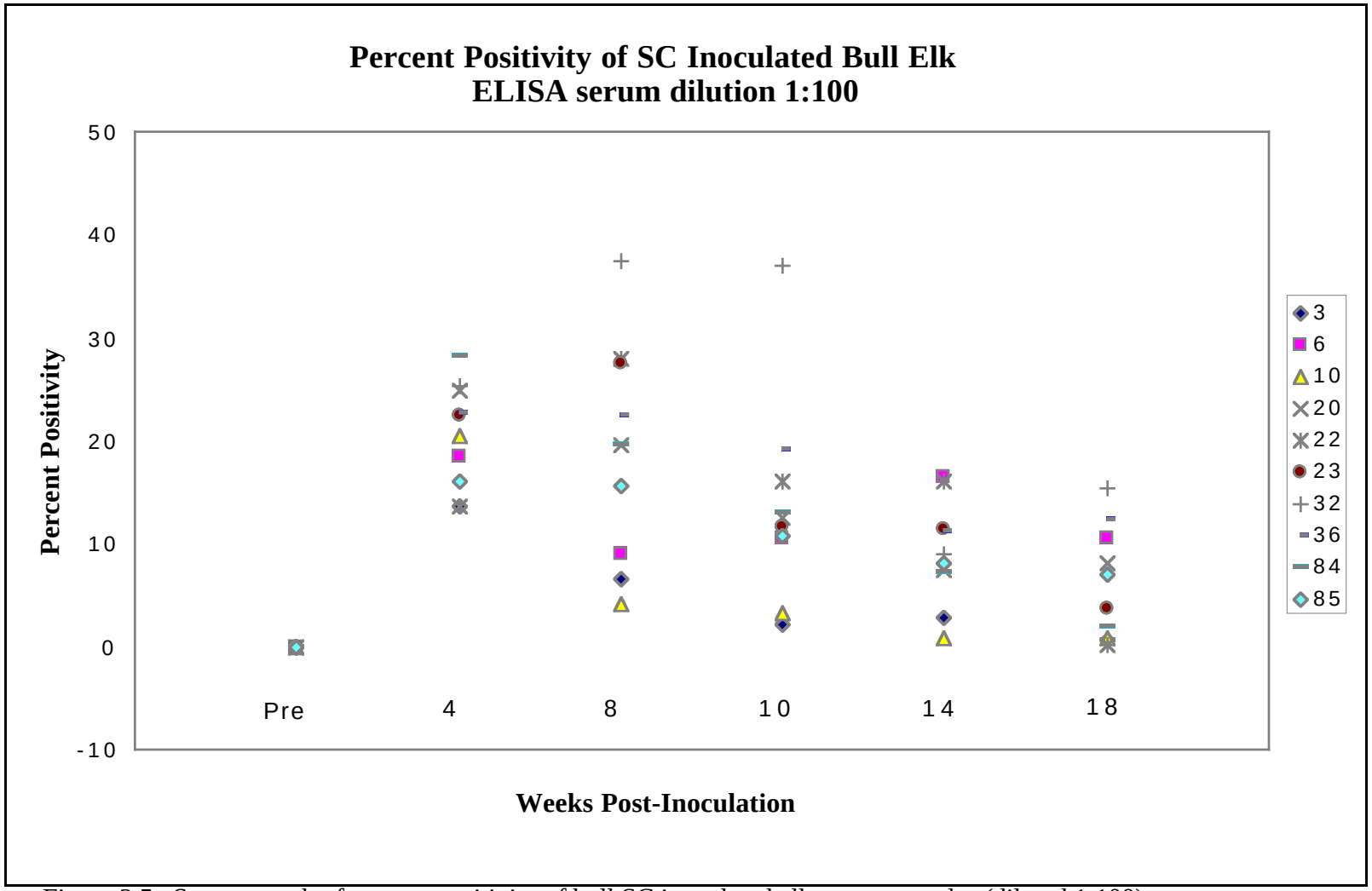

Figure 2.5. Scatter graph of percent positivity of bull SC inoculated elk serum samples (diluted 1:100).

When identical calculations were performed on the 1:50 serum dilution ELISA values all samples from each saline inoculated elk was included within three standard deviations of the mean (2.1 percent positivity) and therefore creation of a suspect range was not necessary. Both the 1:50 and 1:100 serum dilutions show the same overall trends in sample positivity but since the use of the 1:50 dilution did not give suspect readings, the 1:50 dilution in this ELISA was chosen with which to perform all additional analysis.

Table 2.19. Classification of ELISA percent positivity values for elk sera diluted 1:50.

Classification of serum samples	Range of percent positivity
negative	0–2.10%
positive	> 2.11%

Tables 2.20 - 2.23 represent the percent positivity of the 1:50 serum dilution of all elk serum samples in each group as determined by ELISA. Shaded cells represent samples classified as positive based upon a negative cutoff value of 2.1 percent positivity. This negative cutoff value was obtained by averaging and adding three times the standard deviation of the percent positivity values of all post-inoculation samples of saline inoculated elk. Any samples with percent positivity values above the negative cutoff value were considered to have a significant anti-RB51 antibody response.

Table 2.20. Percent positivity of saline inoculated elk as determined by ELISA (serum dilution 1:50)

Elk #	4 Wks PI*	8 Wks PI	10 Wks PI	14 Wks PI	18 Wks PI	27 Wks PI
2	0.00	-0.20	-0.53	1.18	-0.07	0.13
5	0.00	-0.46	-0.15	-0.46	-0.31	0.46
8	1.07	-0.51	-0.21	-0.51	0.24	-0.66
11	-0.82	-0.89	-0.69	-0.89	-1.10	-0.89
24	-0.02	0.45	-0.22	-0.08	-0.22	0.12
31	0.25	-0.25	-0.54	-0.83	-0.54	-0.25
33	0.07	-0.07	-0.07	0.00	0.00	0.14
38	0.33	-2.76	-2.76	-2.76	-2.76	-2.76
50	-0.35	0.02	0.75	-0.35	-0.64	-0.35
53	0.02	0.22	0.02	0.02	-0.12	0.02
55	-0.63	-0.56	-0.49	-0.63	-0.84	-0.63
58	-2.02	-1.96	-2.27	-2.34	-2.27	-2.40
63	0.11	-0.08	-0.02	0.17	0.24	-2.73
66	0.06	0.21	0.06	0.06	-0.25	0.06
74	0.19	0.19	-0.16	-0.09	-0.09	0.05
82	0.01	-0.04	-0.04	-0.10	0.07	-0.16

*approximate weeks post-inoculation

shaded cells represent samples classified as positive based on a negative cutoff value of 2.10% positivity

TABLE 2.21. Percent positivity of ballistically inoculated elk as determined by ELISA (serum dilution 1:50)

Elk #	4 Wks PI*	8 Wks PI	10 Wks PI	14 Wks PI	18 Wks PI	27 Wks PI
4	3.30	6.74	6.64	0.15	-1.17	-1.88
25	3.33	7.44	5.38	6.26	5.68	11.55
27	6.70	10.86	14.51	9.23	6.40	7.07
30	1.90	9.11	10.18	6.87	4.30	0.58
37	18.72	44.94	44.13	25.06	9.74	2.30
40	5.39	15.49	11.75	10.48	14.94	1.41
41	6.82	27.89	14.05	20.50	24.43	8.71
46	14.90	20.51	8.01	10.90	23.40	20.67
47	2.89	12.69	9.06	8.75	18.54	6.27
49	3.13	11.76	9.51	4.63	5.63	4.13
54	5.83	16.28	8.80	5.58	10.41	7.07
59	5.09	11.24	12.31	11.01	7.40	2.84
67	2.84	15.91	12.47	11.95	17.47	13.18
69	3.00	15.21	17.33	4.56	0.64	-1.23
70	7.75	20.54	17.63	11.29	2.05	-0.10
72	6.46	18.74	10.86	9.94	11.09	1.83
76	9.06	17.44	16.11	15.26	18.05	16.35
79	6.51	16.84	38.66	29.19	10.73	1.13

*approximate weeks post-inoculation
shaded cells represent samples classified as positive based on a negative cutoff value of 2.10% positivity

Table 2.22. Percent positivity of subcutaneously inoculated female elk as determined by ELISA (serum dilution 1:50)

Elk #	4 Wks PI*	8 Wks PI	10 Wks PI	14 Wks PI	18 Wks PI	27 Wks PI
7	14.76	3.77	1.96	-1.02	-0.08	-0.86
9	14.99	13.83	12.31	5.07	6.59	0.72
39	31.64	22.17	13.50	2.28	2.37	0.44
43	17.17	15.67	7.85	3.75	4.30	0.28
45	24.98	-2.95	18.13	15.01	10.83	7.02
48	22.77	16.87	18.14	10.65	-0.04	-0.52
56	38.24	26.14	20.85	10.33	8.24	2.75
57	60.38	18.64	12.90	12.25	12.18	10.52
60	24.10	22.07	17.22	17.08	20.59	9.84
61	43.85	5.03	0.18	0.32	0.25	-2.85
62	21.64	17.84	17.45	21.12	23.21	13.65
68	20.37	19.83	16.91	15.18	15.32	13.46
71	19.37	21.29	16.07	19.64	29.40	22.94
75	27.17	18.84	20.43	25.36	26.89	25.99
77	14.92	19.32	22.23	24.66	17.08	18.98
78	17.01	17.93	18.79	15.41	5.05	1.18
81	17.13	8.46	13.79	16.73	21.53	21.66
89	21.16	11.40	16.12	7.59	6.82	3.00
91	10.26	5.04	1.20	0.31	0.10	7.52
99	20.32	19.42	13.75	10.23	14.24	3.46

*approximate weeks post-inoculation
shaded cells represent samples classified as positive based on a negative cutoff value of 2.10% positivity

Table 2.23. Percent positivity of subcutaneously inoculated bull elk as determined by ELISA (serum dilution 1:50)

Elk #	4 Wks PI*	8 Wks PI	10 Wks PI	14 Wks PI	18 Wks PI
3	14.94	12.84	5.09	9.03	1.74
6	18.49	15.26	15.80	19.10	12.49
10	17.95	8.21	8.73	3.26	2.66
20	24.85	21.62	25.60	20.53	20.12
22	22.11	26.59	22.78	22.44	0.17
23	15.93	24.92	10.71	17.09	12.09
32	19.48	36.41	49.27	22.20	29.41
36	19.09	23.35	21.81	22.03	18.51
84	18.59	27.56	21.00	15.51	6.81
85	12.63	9.48	14.17	10.88	10.60

*approximate weeks post-inoculation
shaded cells represent samples classified as positive based on a negative cutoff value of 2.10% positivity

The following figures (figures 2.6 - 2.10) are scatter graphs of the percent positivity of each elk's serum sample within their respective groups. ELISAs were performed with whole cell RB51 antigen, elk sera diluted 1:50, mouse monoclonal anti-bovine IgG_1 and goat anti-mouse IgG (whole molecule). Percent positivity was calculated as: percent positivity =100 X [specific O.D. of test sera / ((positive control O.D.) - (no serum control O.D.))]. Note that the first graph of saline inoculated elk has a smaller y-axis scale. The dashed line at 2.1 % positivity represents the negative cutoff value in this system. The numbers in the box on the right of each figure represent the individual elk identification numbers.

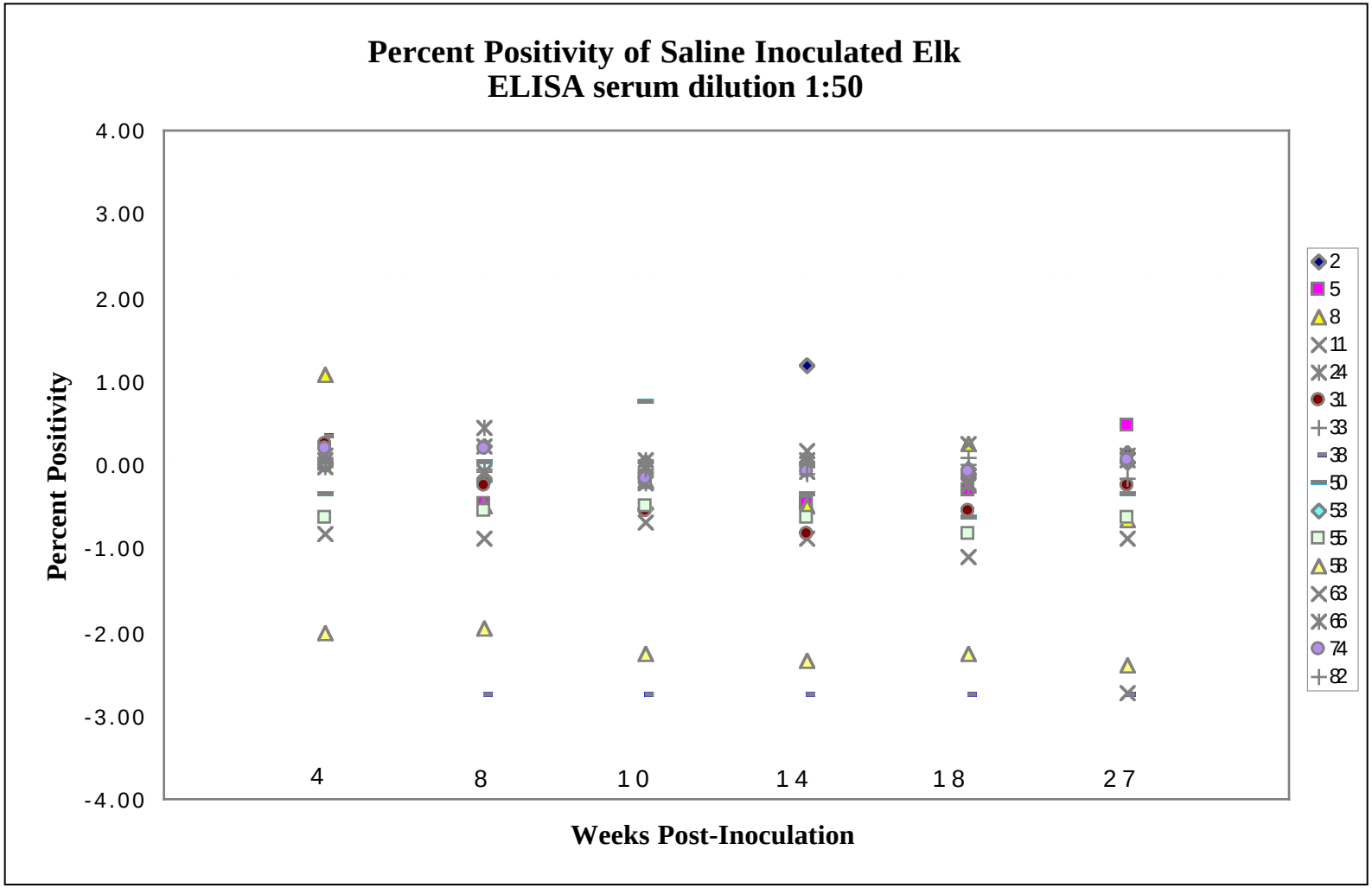

Figure 2.6. Scatter graph of percent positivity of serum samples (diluted 1:50) obtained from all saline inoculated elk.

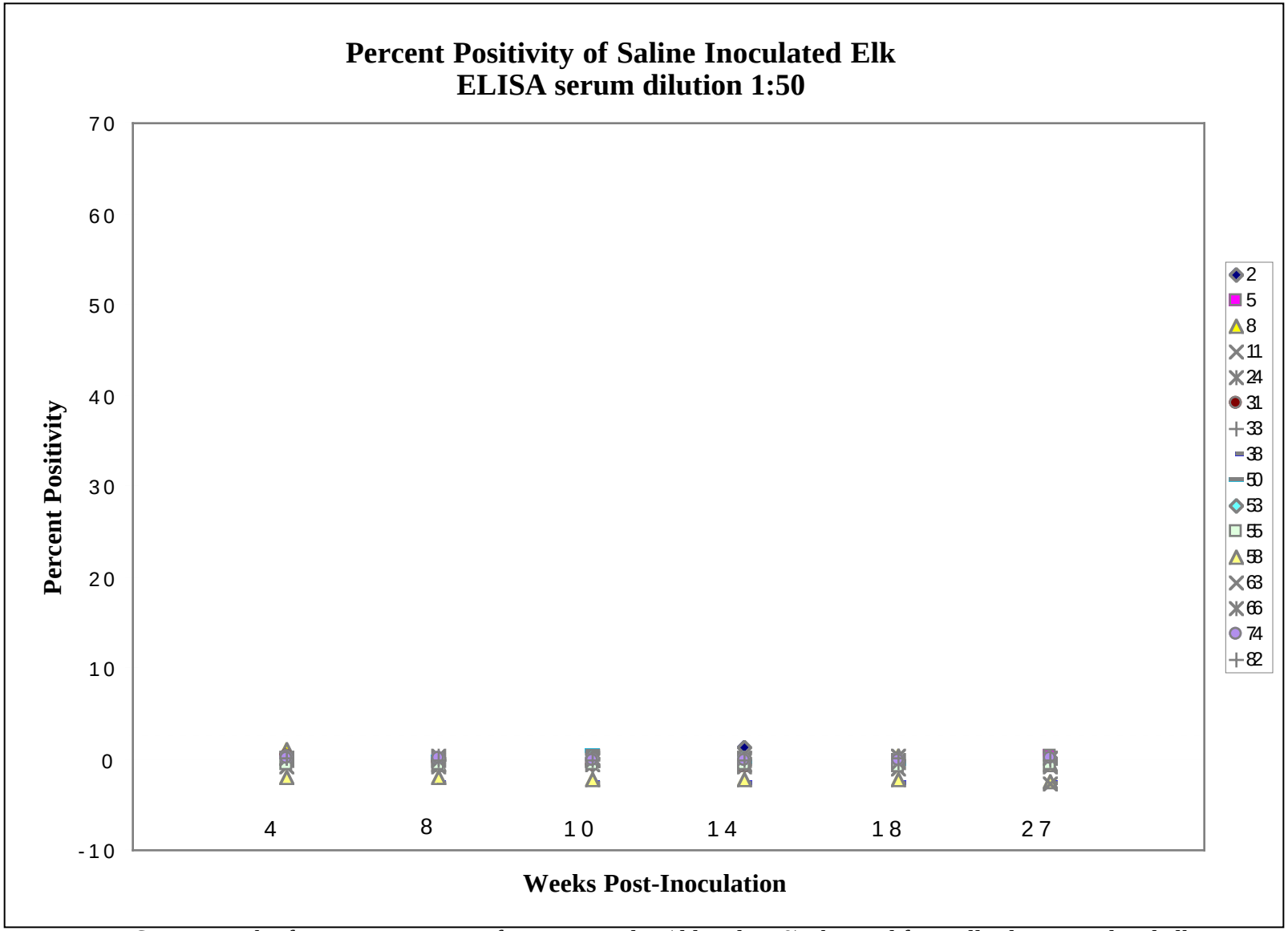

Figure 2.7. Scatter graph of percent positivity of serum samples (diluted 1:50) obtained from all saline inoculated elk.

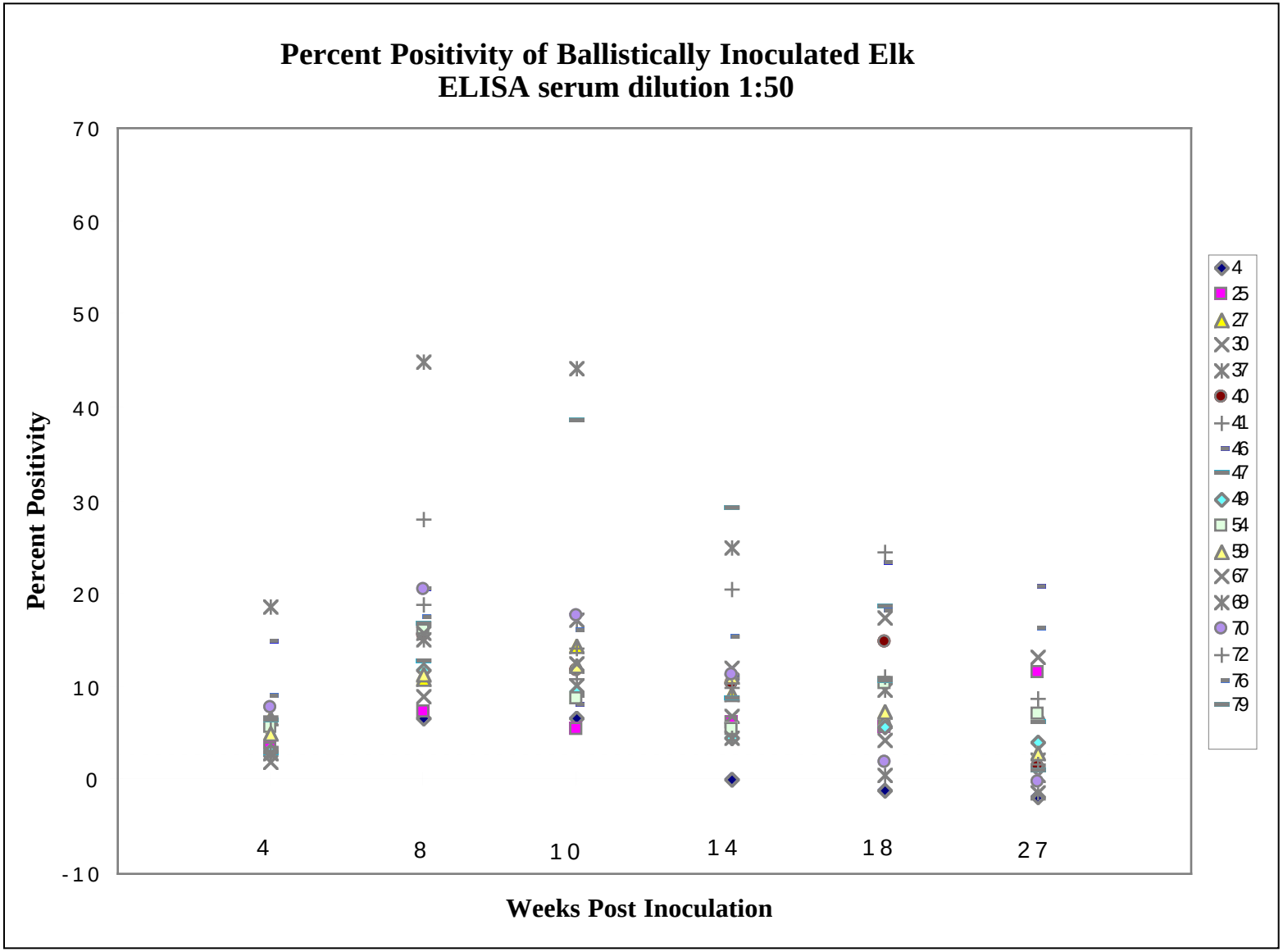

Figure 2.8. Scatter graph of percent positivity of serum samples (diluted 1:50) obtained from all ballistically inoculated elk.

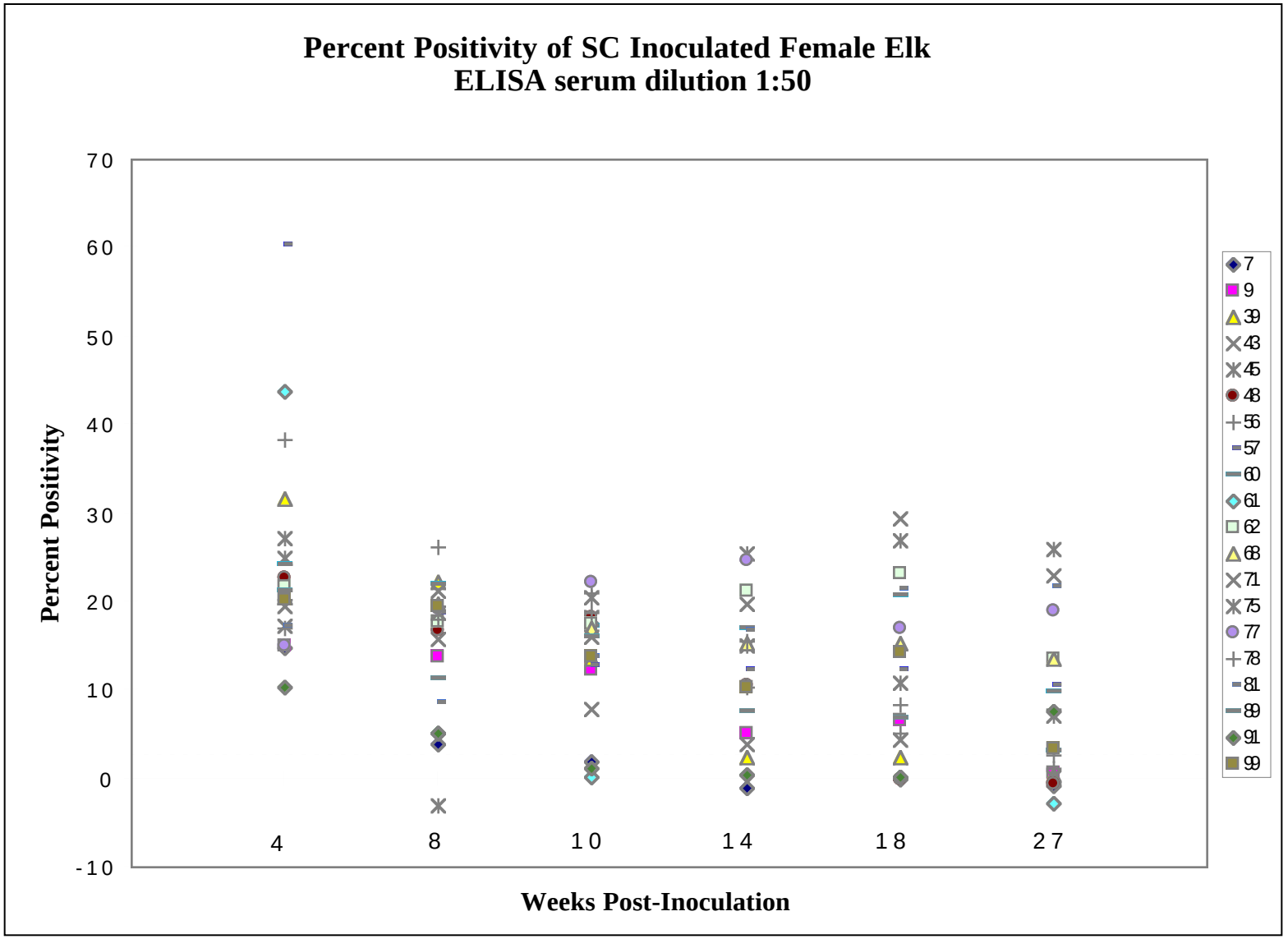

Figure 2.9. Scatter graph of percent positivity of serum samples (diluted 1:50) obtained from all female SC inoculated elk.

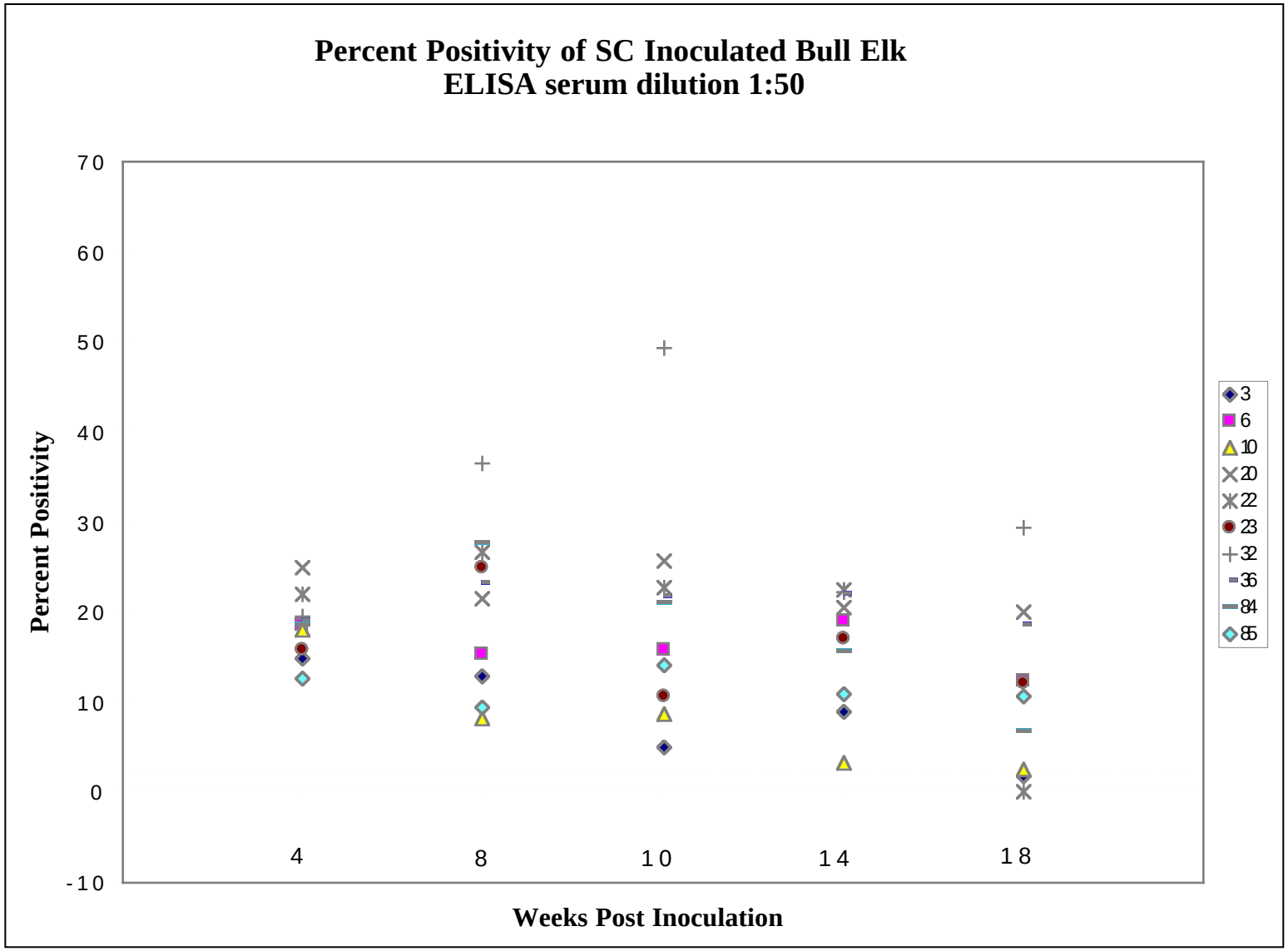

Figure 2.10. Scatter graph of percent positivity of serum samples (diluted 1:50) obtained from all bull SC inoculated elk.

The calculated specific optical density for each elk serum samples within each group are listed in tables 2.24 - 2.27. ELISAs were performed with whole-cell RB51 antigen, elk sera diluted 1:50, mouse monoclonal anti-bovine IgG_1 and horseradish peroxidase conjugated goat anti-mouse IgG (whole molecule). Specific O.D. values were calculated as: (O.D. of test serum) - (average O.D. of all of the elk's pre-inoculation serum samples).If these values are plotted (x axis = weeks post-infection, y axis = specific OD) the resulting graphs have trends similar to the graphs of percent positivity values. However, this analysis precludes comparisons between ELISA plates and is thus less useful than the analysis of percent positivity values.

Table 2.24. Specific O.D. of saline inoculated elk serum samples

Group	Elk #	4 Wks PI Specific OD	8 Wks PI Specific OD	10 Wks PI Specific OD	14 Wks PI Specific OD	18 Wks PI Specific OD	27 Wks PI Specific OD
Saline	2	0.034	-0.005	-0.007	0.002	0.002	0.002
	5	0.002	-0.001	0.003	0.001	0.024	0.028
	8	-0.015	0.043	-0.021	-0.024	-0.025	-0.023
	11	-0.001	0.000	-0.005	-0.001	-0.005	-0.002
	24	0.003	0.006	0.001	-0.003	-0.003	0.003
	31	0.001	0.001	-0.001	-0.003	0.007	0.005
	33	-0.013	0.007	0.005	-0.009	0.002	-0.007
	38	0.000	-0.040	-0.040	-0.040	-0.040	-0.040
	50	-0.001	0.000	-0.002	-0.002	0.000	0.000
	53	0.001	0.004	0.000	0.002	0.008	0.010
	55	-0.004	-0.002	-0.001	0.005	-0.002	-0.001
	58	-0.010	-0.012	-0.012	-0.014	-0.014	-0.012
	63	0.000	0.000	0.000	-0.014	0.004	-0.044
	66	-0.001	-0.001	-0.001	0.001	-0.001	0.002
	74	0.004	0.000	0.000	0.002	0.003	0.004
	82	-0.002	-0.004	-0.005	-0.001	0.000	-0.001

Table 2.25. Specific O.D. of ballistically inoculated elk serum samples

Group	Elk #	4 Wks PI Specific OD	8 Wks PI Specific OD	10 Wks PI Specific OD	14 Wks PI Specific OD	18 Wks PI Specific OD	27 Wks PI Specific OD
Biobullet	4	0.019	0.128	0.039	0.013	0.005	0.007
	25	0.084	0.082	0.020	0.031	0.020	0.033
	27	0.110	0.170	0.173	0.083	0.060	0.038
	30	0.022	0.136	0.048	0.030	0.014	0.002
	37	0.165	0.567	0.537	0.207	0.062	0.013
	40	0.031	0.169	0.115	0.091	0.086	0.004
	41	0.047	0.391	0.177	0.189	0.201	0.037
	46	0.231	0.327	0.113	0.129	0.241	0.224
	47	0.032	0.178	0.174	0.126	0.244	0.032
	49	0.022	0.223	0.171	0.033	0.045	0.021
	54	0.053	0.220	0.141	0.069	0.133	0.038
	59	0.044	0.233	0.232	0.158	0.088	0.012
	67	0.025	0.328	0.245	0.235	0.313	0.116
	69	0.065	0.292	0.258	0.035	0.004	-0.002
	70	0.126	0.342	0.311	0.070	0.015	-0.002
	72	0.110	0.372	0.236	0.183	0.106	0.014
	76	0.108	0.302	0.251	0.218	0.188	0.149
	79	0.067	0.333	0.439	0.347	0.081	0.005

Table 2.26. Specific O.D. of SC inoculated female elk serum samples

Group:	Elk #	4 Wks PI Specific OD	8 Wks PI Specific OD	10 Wk Specific OD	14 Wks PI Specific OD	18 Wks PI Specific OD	27 Wks PI Specific OD
Female SQ	7	0.233	0.032	0.025	0.001	0.009	0.003
	9	0.609	0.231	0.097	0.019	0.021	0.002
	39	0.432	0.252	0.104	0.008	0.005	0.001
	43	0.260	0.158	0.042	0.022	0.027	0.006
	45	0.278	-0.040	0.081	0.031	0.024	0.015
	48	0.317	0.148	0.132	0.054	-0.005	-0.002
	56	0.505	0.332	0.201	0.093	0.043	0.015
	57	0.606	0.214	0.081	0.103	0.096	0.046
	60	0.267	0.237	0.163	0.171	0.224	0.021
	61	0.536	0.004	0.001	0.000	0.004	-0.040
	62	0.307	0.248	0.229	0.229	0.279	0.100
	68	0.240	0.178	0.166	0.111	0.014	-0.010
	71	0.280	0.312	0.223	0.261	0.376	0.194
	75	0.402	0.280	0.322	0.338	0.373	0.221
	77	0.258	0.347	0.347	0.313	0.242	0.215
	78	0.376	0.374	0.270	0.154	0.028	0.010
	81	0.325	0.113	0.117	0.179	0.213	0.222
	89	0.285	0.088	0.089	0.028	0.023	0.013
	91	0.118	0.028	0.009	0.001	0.000	0.056
	99	0.336	0.231	0.154	0.137	0.139	0.019

Table 2.27. Specific O.D. of SC inoculated bull elk serum samples

Group:	Elk #	4 Wks PI Specific OD	8 Wks PI Specific OD	10 Wks PI Specific OD	14 Wks PI Specific OD	18 Wks PI Specific OD
Bull SQ	3	0.206	0.101	0.031	0.041	0.008
	6	0.240	0.116	0.136	0.214	0.138
	10	0.274	0.055	0.042	0.011	0.010
	20	0.362	0.286	0.183	0.110	0.120
	22	0.204	0.417	0.241	0.241	0.002
	23	0.309	0.378	0.161	0.156	0.050
	32	0.354	0.525	0.518	0.125	0.215
	36	0.308	0.307	0.262	0.154	0.168
	84	0.421	0.293	0.194	0.109	0.027
	85	0.229	0.223	0.155	0.117	0.099

All 1:50 ELISA sample data within each group was evaluated by repeated measures analysis in Proc GLM (SAS Version 6.0 4th edition - Cary, Inc.) to determine when (of the six post-inoculation serum collection times) elk are most likely to produce a detectable antibody response. Table 2.28 lists the p-values calculated through repeated measures comparing average percent positivity values between consecutive samples within a group. A p-value less than 0.005 represents a significant difference between bleeds.

TABLE 2.28. P - values calculated through repeated measures comparing percent positivity values between consecutive samples within a group

Experimental group	4 wks PI vs 8 wks PI	8 wks PI vs 10 wks PI	10 wks PI vs 14 wks PI	14 wks PI vs 18 wks PI	18 wks PI vs 27 wks PI
Saline inoculated	(0.1772)	(0.6433)	(0.8968)	(0.5307)	(0.7304)
Ballistically inoculated	0.0001	(0.3269)	(0.0173)	(0.7259)	0.0014
Female SC inoculated	0.0049	(0.4868)	(0.0370)	(0.7501)	0.0030
Male SC inoculated	(0.3999)	(0.6478)	(0.2953)	(0.0829)	

( ) indicates p-values > 0.005
gray shaded cells indicate p-values < 0.005.

Analysis showed that percent positivity values within the saline group (which were never vaccinated with RB51 bacteria) did not significantly vary over time ($p > 0.05$). All other groups were shown to have a statistically significant variance of percent positivity values over time. The female and male subcutaneously inoculated animals had their highest mean percent positivity value at four weeks PI and eight weeks PI respectively. The mean value of the female group at four weeks PI (24.1% positivity) was statistically distinct from the value at eight weeks PI (15.0%

positivity) ($p = 0.0049$). The bull group's mean percent positivity value at four weeks PI (18.4% positivity) was not statistically different ($p = 0.3999$) from the mean value at eight weeks PI (20.6% positivity). The lack of significance between the four and eight weeks PI are likely due to the small size of the experimental bull group as well as a very large variance among samples for each time period within the bull group.

The biobullet group's highest mean value (16.6% positivity) was not achieved until eight weeks PI. This mean value was statistically distinct from the mean value at four weeks PI ($p = 0.001$). Mean values for all groups steadily declined after eight weeks PI.

Elk Dotblot Results

Due to the lack of staining (color) density uniformity within each dot and the extreme sensitivity of the NIH Image program, standard deviations of the individual density measurements (pixel readings) in each dot area were often very large. Regardless of the level and variability of color development in the center-most of a dot, a thin dark circle often formed at the perimeter of the dot making interpretation even more difficult. Also, samples found to have high antibody titers by ELISA often produced dots whose pixel readings had extremely large standard deviations. Although the NIH image program gave objective quantitation of a dot's average density, the large variation of pixel density for each dot did not permit determination of statistically significant differences. Since this method of quantitation was highly time consuming and could not yield statistically tight values, it was discontinued. The results obtained with two elk are tabulated below to illustrate the quantification problems.

TABLE 2.29. Dotblot results with sera of female ballistically inoculated elk #54 using NIH Image program

Group	Elk #	Weeks post inoculation	Pixel density (serum dilution 1:20)	Average pixel density of two 1:20 serum dilutions	SD* within sample area	Pixel density (serum dilution 1:100)	Average pixel density of two 1:100 serum dilutions	SD within sample area
Biobullet	54	Prevaccination	2.02	1.24	3.46	26.94	26.29	11.18
			0.46		1.36	25.64		10.71
		at Vaccination	0.06	1.07	0.32	0.43	1.21	1.13
			2.07		8.24	1.99		3.36
		4	20.27	15.38	11.85	8.23	4.725	8.11
			10.49		7.9	1.22		2.25
		8	23.22	25.69	10.82	5.25	5.9	4.3
			28.16		8.2	6.55		5.13
		10	15.98	16.39	8.71	2.34	2.125	3.38
			16.8		8.85	1.91		2.92
		14	25.57	29.22	11.7	10.93	12.51	7.44
			32.87		12.1	14.09		7.25
		18	36.09	32.74	14.74	16.94	17.105	6.75
			29.39		12.17	17.27		7.96
		27	9.26	9.96	5.5	1.77	1.84	2.47
			10.65		4.56	1.91		3.06

* standard deviation

TABLE 2.30. Dotblot results with sera of saline inoculated elk #2 using NIH Image program

Group	Elk #	Weeks post inoculation	Pixel density (dilution 1:20)	Average pixel density of two 1:20 dilutions	SD* within sample area	Pixel density (dilution 1:100)	Average pixel density of two 1:100 dilutions	SD within sample area
Saline	2	Prevaccination	0.09	0.15	0.4	0.6	0.42	1.44
			0.21		0.84	0.23		0.71
		at Vaccination	2.12	4.51	3.56	6.41	6.5	6.49
			6.89		7.17	6.58		6.26
		4	8.02	7.25	6.41	6.95	3.93	6.42
			6.48		5.74	0.91		1.82
		8	0.92	1.63	1.61	1.32	1.88	1.99
			2.34		3.85	2.43		2.86
		10	3.47	2.04	2.98	4.23	2.74	2.88
			0.61		1.28	1.24		2.61
		14	3.39	3.01	4.24	0.81	0.89	1.67
			2.62		3.77	0.96		1.8
		18	0.29	0.46	1.21	1.35	0.96	2.56
			0.62		1.72	0.57		1.48
		27	1.45	1.78	2.81	2.22	4.81	2.61
			2.11		3.72	7.4		5.58

* standard deviation

All dotblots were visually evaluated by the author. Dots from samples were visually compared to both prevaccination dots (negative controls) and Steer 66 dots (positive control) and then classified as either positive or negative. Tables 2.31 - 2.34 are a compilation of ELISA and visual dotblot results of all animals. The percent of animals positive within each group for each test is listed below its respective table.

TABLE 2.31. Comparison of serum samples results within saline inoculated elk group based on percent positivity in ELISA and visual evaluation of dotblots (serum dilution 1:50)

Group	Elk #	4 Wks PI		8 Wks PI		10 Wks PI		14 Wks PI		18 Wks PI		27 Wks PI	
		Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot
Saline	2	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-	-	-	-	-
	31	-	-	-	-	-	-	-	-	-	-	-	-
	33	-	-	-	-	-	-	-	-	-	-	-	-
	38	-	+	-	+	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-	-	-	-	-
	53	-	-	-	-	-	-	-	-	-	-	-	-
	55	-	+	-	-	-	-	-	-	-	-	-	-
	58	-	-	-	-	-	-	-	-	-	-	-	-
	63	-	-	-	-	-	-	-	-	-	-	-	-
	66	-	-	-	-	-	-	-	-	-	-	-	-
	74	-	-	-	-	-	-	-	-	-	-	-	-
	82	-	-	-	-	-	-	-	-	-	-	-	-
% Positive		**0%**	**12.5%**	**0%**	**6.25%**	**0%**	**0%**	**0%**	**0%**	**0%**	**0%**	**0%**	**0%**

note: shaded areas indicate samples with differing ELISA and dotblot results

TABLE 2.32. Comparison of serum samples within ballistically inoculated elk group based on percent positivity in ELISA and visual evaluation of dotblots (serum dilution 1:50)

Group	Elk #	4 Wks PI		8 Wks PI		10 Wks PI		14 Wks PI		18 Wks PI		27 Wks PI	
		Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot
Biobullet	4	+	+	+	+	+	+	-	-	-	-	-	-
	25	+	+	+	+	+	+	+	+	+	+	+	+
	27	+	+	+	+	+	+	+	+	+	+	+	+
	30	-	+	+	+	+	+	+	+	+	+	-	-
	37	+	+	+	+	+	+	+	+	+	-	+	-
	40	+	+	+	+	+	+	+	+	+	+	-	-
	41	+	-	+	+	+	+	+	+	+	+	+	-
	46	+	+	+	+	+	+	+	+	+	+	+	+
	47	+	-	+	+	+	+	+	+	+	+	+	-
	49	+	-	+	+	+	+	+	+	+	-	+	-
	54	+	+	+	+	+	+	+	+	+	+	+	-
	59	+	+	+	+	+	+	+	+	+	+	+	+
	67	+	-	+	+	+	-	+	-	+	-	+	-
	69	+	+	+	+	+	+	+	+	-	-	-	-
	70	+	+	+	+	+	+	+	-	-	-	-	-
	72	+	+	+	+	+	+	+	+	+	+	-	-
	76	+	+	+	+	+	+	+	+	+	+	+	+
	79	+	+	+	+	+	+	+	+	+	+	-	-
% Positive		**94.4%**	**77.8%**	**100%**	**100%**	**100%**	**94.4%**	**94.4%**	**83.3%**	**83.3%**	**66.7%**	**61.1%**	**27.8%**

note: shaded areas indicate samples with differing ELISA and dotblot results

TABLE 2.33. Comparison of serum samples within SC inoculated female elk group based on percent positivity in ELISA and visual evaluation of dotblots (serum dilution 1:50)

Group	Elk #	4 Wks PI		8 Wks PI		10 Wks PI		14 Wks PI		18 Wks PI		27 Wks PI	
		Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot
Female SQ	7	+	+	+	+	-	-	-	-	-	-	-	-
	9	+	+	+	+	+	+	+	+	+	-	-	-
	39	+	+	+	+	+	+	+	+	+	-	-	-
	43	+	+	+	+	+	+	+	-	+	-	-	-
	45	+	+	-	+	+	+	+	+	+	+	+	+
	48	+	+	+	+	+	+	+	-	-	-	-	-
	56	+	+	+	+	+	+	+	-	+	-	+	-
	57	+	+	+	+	+	+	+	+	+	+	+	-
	60	+	+	+	+	+	+	+	+	+	+	+	-
	61	+	-	+	-	-	-	-	-	-	-	-	-
	62	+	+	+	+	+	+	+	+	+	+	+	-
	68	+	+	+	+	+	+	+	+	+	+	+	+
	71	+	+	+	+	+	+	+	+	+	+	+	+
	75	+	+	+	+	+	+	+	+	+	+	+	+
	77	+	+	+	+	+	+	+	+	+	+	+	+
	78	+	+	+	+	+	+	+	+	+	-	-	-
	81	+	+	+	-	+	+	+	+	+	+	+	+
	89	+	+	+	-	+	-	+	-	+	-	+	-
	91	+	+	+	+	-	-	-	-	-	-	+	-
	99	+	+	+	+	+	+	+	+	+	+	+	-
% Positive		**100%**	**95%**	**95%**	**85%**	**85%**	**80%**	**85%**	**65%**	**80%**	**45%**	**65%**	**30%**

note: shaded areas indicate samples with differing ELISA and dotblot results

TABLE 2.34. Comparison of serum samples within SC inoculated Bull elk group based on percent positivity in ELISA and visual evaluation of dotblots (serum dilution 1:50)

Group	Elk #	4 Wks PI		8 Wks PI		10 Wks PI		14 Wks PI		18 Wks PI	
		Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot	Elisa	Dotblot
Bull SQ	3	+	+	+	+	+	+	+	+	-	-
	6	+	+	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	-	+	-
	20	+	+	+	+	+	+	+	+	+	+
	22	+	+	+	+	+	+	+	+	-	-
	23	+	+	+	+	+	+	+	+	+	+
	32	+	+	+	+	+	+	+	+	+	+
	36	+	+	+	+	+	+	+	+	+	+
	84	+	+	+	+	+	+	+	+	+	-
	85	+	+	+	+	+	+	+	+	+	+
% Positive		100%	100%	100%	100%	100%	100%	100%	90%	80%	60%

note: shaded areas indicate samples with differing ELISA and dotblot results

Western Blotting of Elk Serum Samples

Below (Figures 2.11 - 2.28) are links to digital images of selected elk serum samples (collected before and after vaccination with RB51) reacted with RB51 nitrocellulose strips. The post-inoculation serum samples selected for each elk was the one which gave the highest percent positivity value among all bleeds from that animal when tested in ELISA at a 1:50 serum dilution. The first strip of each figure (sheet) is the broad range molecular weight standard (MW). The next 2 strips depict the reaction with the pre- and post-vaccination serum sample from an individual animal. With the exception of the MW strip, this pattern is repeated for two additional elk. The same nitrocellulose sheets developed first with monoclonal mouse anti-bovine IgG_1 and then with polyclonal rat anti-mouse IgG (whole molecule) are pictured for each animal.

Figure 2.11. Western blot of saline elk #2 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

Figure 2.12. Western blot of saline elk #2 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

Figure 2.13. Western blot of saline elk #24, 31, and 50 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

Figure 2.14. Western blot of saline elk #24, 31, and 50 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

Figure 2.15. Western blot of saline elk #53 and 55 biobullet elk #76 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

Figure 2.16. Western blot of saline elk #53 and 55 biobullet elk #76 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

Figure 2.17. Western blot of biobullet elk #4, 27, and 47 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

Figure 2.18. Western blot of biobullet elk #4, 27, and 47 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

Figure 2.19. Western blot of biobullet elk #54, 76, and 79 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

Figure 2.20. Western blot of biobullet elk #54, 76, and 79 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

Figure 2.21. Western blot of female subcutaneously inoculated elk #43, 68, and 78 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

Figure 2.22. Western blot of female subcutaneously inoculated elk #43, 68, and 78 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

Figure 2.23. Western blot of female subcutaneously inoculated elk #81, 91, and 99 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

Figure 2.24. Western blot of female subcutaneously inoculated elk #81, 91, and 99 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

Figure 2.25. Western blot of bull subcutaneously inoculated elk #6, 32, and 36 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

Figure 2.26. Western blot of bull subcutaneously inoculated elk #6, 32, and 36 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

Figure 2.27. Western blot of bull subcutaneously inoculated elk #20 and 84 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

Figure 2.28. Western blot of bull subcutaneously inoculated elk #20 and 84 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

Essentially all elk sera (pre- and post-vaccination) demonstrated reactivity with one or more antigens if the polyclonal anti-bovine reagent was used to develop the reactions. The antigens ranged from 17 – 70 kDa. Therefore, it was not possible to identify antigen - antibody reactions specifically due to the RB51 exposure using the polyclonal anti-bovine IgG reagent.

In general, the pre-immunization serum samples from all elk (except elk #68) and all the serum samples from the saline inoculated elk with the exception of elk #53 at 8 weeks PI, did not have IgG_1 antibodies to antigen ranging from 17 – 70 kDa. A few elk from the RB51 inoculated elk demonstrated an IgG_1 reaction within the antigen range.

All post - RB51 inoculated elk serum samples recognized IgG1 antibodies to one or two bands in the 6 to 17 kDa range. These bands were not observed with any elk sera obtained prior to vaccination or from saline inoculated elk.

TABLE 2.35. Number of bands noted between 6 and 17 kDa for each elk post-inoculation serum sample* tested in Western blot.

Elk Group:	Elk #	Number of bands between 6 and 17 kDa
Saline	2	0
	24	0
	31	0
	50	0
	53	0
	55	0
Ballistically inoculated	4	1
	27	1
	47	1
	54	1
	76	1
	79	1
Female SC inoculated	43	2
	68	2
	78	2
	81	1
	91	1
	99	2
Bull SC inoculated	6	3
	32	2
	36	2
	84	1

*elk serum samples tested were those that yielded the highest percent positivity values in ELISA (1:50 serum dilution) for that elk.

At times, it was difficult to decide if some thick bands were actually multiple or single bands.

DISCUSSION

Identification of Elk Isolates

All bacteriological and biochemical tests indicated that every viable elk culture sent to our laboratory was *B. abortus* strain RB51. No differences could be found between the elk isolates and the original inoculums, indicating that RB51 is **stable** during passage through elk. This agrees with the observations in mice (106). These results also indicate that processing RB51 into lactose-based pellets that are ballistically delivered to elk does not significantly alter the biochemical characteristics of RB51.

Analysis of Elk ELISAs

After evaluating serum samples in several different tests and with multiple ELISA data analyses, the ELISA was found to be the most accurate and easily reproducible method as well as the most convenient to identify elk with RB51 specific titers. As with any test, interpretation of results is the most important as well as the most difficult step.

For results from a test to be considered valid, appropriate controls must be evaluated. Numerous controls were utilized in the ELISA developed for detection of specific anti-RB51 antibodies in elk serum. Non-specific binding of antibodies to the polystyrene plate or the RB51 antigen was evaluated in wells containing either no antigen coating or no elk serum. A high level of non-specific binding in any of these wells would result in a elevated optical density value. After extensive testing it was concluded that both the polyclonal and monoclonal antibodies used in this ELISA did not bind non-specifically to the plate, antigen, or to the elk sera. As a result, these reagents were not routinely evaluated for non-specific binding. Duplicates of serum samples obtained prior to vaccination (before vaccination and on the day of vaccination) were assumed to contain no specific antibodies to RB51 and were used as negative controls for each animal in each plate. The magnitude and variability of the optical density value obtained with these samples was assumed to represent the normal 'background' value for the animal. Since no test had previously been developed to assess the level of anti-RB51 antibodies in elk serum, an elk serum of known antibody level was not available. As a result, a bovine serum (Steer 66) which had been repeatedly injected intramuscularly with killed RB51 and had developed specific anti-RB51 antibodies was used as the positive control in each plate. In the future, hyper-immune elk sera should be produced and used as positive control sera.

Many methods for interpreting ELISA data have been described(89, 55, 59). Three methods of data analysis that appear frequently in the literature involve specific optical densities (OD), straight comparisons between pre- and post-inoculation samples, and percent positivities (89). Each of these methods have major limitations. Specific optical densities (calculated as OD of a post-inoculation test serum - OD of the pre-inoculation test serum) do not provide for inter-plate variability. As a result, only samples tested on the same plate can be compared thus precluding use in large study groups. To analyze the specific optical densities, most investigators assign an arbitrary value above which a sample would be considered positive. This value usually has no statistical validity.

Straight comparisons between pre- and post- inoculation values do not allow for comparisons between animals or between plates. Often negative cutoff values are chosen arbitrarily or are based upon a single sample per animal (for example the average plus three times the standard deviation of multiple duplicate wells of the animal's pre-inoculation serum sample).

Percent positivity is a ratio of the specific OD of the sample to the OD of a positive control. It is designed to represent where a sample lies along a straight continuum with zero binding of antibody being equivalent to 0% positivity and the antibody binding from the positive control being equivalent to 100% positivity. Therefore, the percent positivity of each sample represents the ratio of specific antibody in that sample to the amount of specific antibody in the positive control. Since percent positivity is a ratio, the calculation of the positive control serum's percent positivity value technically is not important as long as the same calculation is used for every plate's positive control serum value. However, since plate and animal variability can affect the amount of non-specific binding one could attempt to correct this by subtracting the prevaccination OD of the positive control animal from the post-vaccination sample used as the positive control. This would remove the 'background' of the positive control value and represent where that sample lies along the 0% to 100% positivity continuum.

As a result, I chose to analyze my ELISA's with the slightly modified percent positivity calculation:

$$\text{Percent positivity} = \frac{\text{specific OD of the test serum}}{\text{specific OD of the positive control serum}}$$

where:

$$\text{specific OD}_{\text{test serum}} = \text{OD}_{\text{test serum}} - \text{OD}_{\text{prevaccination serum from the test animal}}$$

$$\text{specific OD}_{\text{positive control serum}} = \text{OD}_{\text{positive control serum}} - \text{OD}_{\text{prevaccination sample of the positive control animal}}$$

Unfortunately, no prevaccination sample from the positive control animal (steer 66) was available. Therefore, the OD value from wells on each plate which were not incubated with the primary serum were substituted for the OD of the prevaccination sample of the positive control animal. Alternatively, a randomly selected, negative bovine sera could have been used. Since each sample is adjusted to both a negative and a positive control tested on the same plate, inter-plate and inter-animal variability is minimized between samples. Since all serum samples were tested in duplicate, inter-well variability was also decreased.

Due to the inclusion of a saline inoculated elk group, a percent positivity value (the negative cutoff value) above which a sample would be considered to have a significantly elevated anti-RB51 antibody level can be calculated. All samples from the saline inoculated elk selected for this study should have no specific anti-RB51 antibodies. As a group, saline inoculated elk would represent many negative samples obtained over the full length of the experiment. Their readings would reflect sample variability due to any uncontrolled conditions that the elk may have been exposed to over time. A negative cutoff value can be computed by averaging the percent positivity of all saline elk samples and adding three times the standard deviation. Using this negative cutoff value, >99.5% of all negative samples in the population which the saline group represents should be correctly identified as negative. Since the saline and the RB51 vaccinated groups belong to the same population, the saline calculated cutoff value should correctly identify >99.5% of all truly negative samples within the RB51 vaccinated groups. As a result, all samples with a percent positivity value over this negative cutoff value are considered to have an appreciable anti-RB51 antibody response. Using this rationale, all RB51 inoculated elk were shown to have an antibody response to vaccination.

Often, managers wish to confirm that animals were successfully vaccinated and are immunologically responding to vaccination. Ideally, confirmation would be achieved soon after vaccination so that the manager could then either revaccinate or remove the non-responding animal from the herd. Therefore, it would be helpful to know the earliest sampling time which would reliably detect an antibody response using the ELISA for RB51 vaccinated animals. Repeated measure analysis suggests that saline inoculated animals could be tested at any time between four and twenty-seven weeks PI as they will all yield negative test values during these times.

When considering the six times that the animals were tested after inoculation, the sample period in which the largest proportion of vaccinated animals can be detected by the ELISA is at 4 wks PI for both the female and male subcutaneously inoculated groups. It may be possible to obtain equally high (or higher) mean percent positivity values before four weeks PI however this cannot be determined from these experiments.

When given the options of testing RB51 biobullet inoculated animals at 4, 8, 10, 14, 18, or 27 weeks PI, the sample period in which the largest proportion of vaccinated animals can be detected by the ELISA is at eight weeks PI. The average percent positivity value rose dramatically between four and eight weeks PI (6.3% positivity to 16.6% positivity) and serum samples taken between four and eight weeks PI may provide either higher or non-significantly different values when compared to the value obtained at eight weeks PI. The delay in reaching maximal values as compared to the female and bull elk vaccinated subcutaneously inoculated group could be attributed to the time required for the biobullet to dissolve allowing the RB51 organisms to contact the

animal's tissues. Alterations of the organism during the processing or firing could cause a delay in antibody response but, no evidence of this has been documented.

The average percent positivity values for the ballistically inoculated group (which was vaccinated with fewer organisms than were the subcutaneously inoculated groups) was consistently lower than for the two subcutaneously inoculated groups. However due to large standard variations within each group, these differences were not statistically significant. This suggests that processing RB51 into lactose based pellets and shooting them into elk (in this system) does not affect the antibody response

The ELISA developed for this project is relatively easy to perform and could likely be developed into an automated, standardized test. It appears to correctly identify inoculated animals without misclassifying negative saline inoculated elk samples. Evaluating the true sensitivity and specificity of the ELISA described in this thesis is, however, impossible as no other method of testing elk serum for anti-RB51 antibodies has been developed.

The use of monoclonal IgG_1 antisera was chosen for use in the ELISA based upon Henning et al.'s (60) finding of 100% cross-reactivity between elk and bovine IgG_1 and upon the finding that field strain *Brucella* infections cause a large IgG_1 antibody response. Use of this anti-sera will only identify antibodies of the IgG_1 sub-isotype. While this is not a true representation of the overall antibody response, it does fulfill the objective of developing an ELISA able to identify RB51 vaccinated elk through serology. Other anti-isotypes may be equally effective for use in the ELISA. However, anti-bovine isotypes other than anti-IgG_1 do not show 100% cross- reactivity.

Researchers associated with the University of Wyoming had reported serological reactions in the card agglutination test with the elk serum after ballistic exposure suggesting that the RB51 organisms processed in the lactose-based pellets had reverted to a smooth form of Brucella. However, bacteriological examination of organisms removed from pellets as well as all isolates recovered from ballistically inoculated elk failed to support this hypothesis. Also, mice experiments (Chapter 3) further indicate the absence of O-chain in the biobullet-derived RB51. No serological testing of these elk's samples for anti-O antibodies was performed as that was beyond the scope of this project. It is likely that any LPS associated O-side chain antibodies detected by the card test would have been the result of LPS contamination of the equipment in which the RB51 organisms were grown. The institution that produced the RB51 organisms used the same fermentor previously used to cultivate smooth *Brucella* organisms. Unpublished data (personal communication - Dr. G. Schurig) indicates that LPS associated O-side chain produced by smooth *Brucella spp* can remain bound to the fermentors after many washings and after autoclaving. Due to the extreme antigenicity of O-side chains, a very low contamination level of a rough organism with the O-chain could still cause a detectable antibody response in vaccinated animals.

Analysis of Elk Dotblots

The dotblot was developed concurrently with the indirect ELISA so that it could be used if the ELISA test was unsuccessful. Analyzing dotblots by use of an optical computer program (NIH Image) was found to be unsatisfactory. Due to the lack of color uniformity within each dot and the extreme sensitivity of the NIH Image program, standard deviations of the measured areas were often very large. Often, regardless of the level of color development in the center-most of a dot, a thin dark circle formed at the perimeter of the dot. This color is likely a precipitate caused by reagents that could not be completely washed out of each well. Also, dots of samples found to have high antibody titers by ELISA often had an extremely large standard deviation in their pixel readings per dot. This may be due to a variety of reasons such as partial drying of the nitrocellulose during the test, or a slight angulation of the dotblot apparatus. Although the NIH

image program allowed for the objective quantitation of dot color density, the large variation of average pixel density for each blot did not permit determination of statistically significant differences.

Although the circular image area was chosen to be slightly smaller than the dots (in an attempt to avoid the circular precipitation) a large variation was found between measurements of the same well. A program supplement (a macro) could be written to have the Image program read a set area in the exact same 96 locations of each dotblot thus decreasing the variability caused by manually placing the image selection area. However, due to the large standard deviation within each well the proper placement of the image selection which would give a true representation of a sample's reactivity is impossible to determine. Therefore, the validity of computer measured optical densities for description of dotblot results appear highly questionable in the presence of strong color variability within each dot.

When considering visually evaluated dotblots, result differences occur most commonly with samples which have a low positive value (2.75 to 13.65 percent positivity) by ELISA. These samples often appear negative in the dotblot, yet the dotblot does not consistently fail to identify positive samples in this range. This discrepancy may be due to low sensitivity of the dotblot as well as human error in visually interpreting each sample result. In addition, five samples identified as negative with ELISA were interpreted as positive with the dotblot. This could also be attributed to human error or could be the result of non-specific binding of serum and/or reagents. Finally, one sample with a high percent positivity in ELISA was determined to be negative by the dotblot. This could be the result of an erroneously high ELISA reading (as the progressive samples from this particular animal quickly became negative) or a result of prozoning in the dotblot.

Overall, the dotblot appears to be less accurate or at least harder to interpret than the ELISA. Unless an automated method is developed for result interpretation the dotblot will continue to produce only highly subjective results. Also, the dotblot protocol and/or equipment will need to be modified such that dots of uniform densities are produced and peripheral reaction rings are avoided.

In addition to difficulties interpreting results, dotblots are not efficient for testing large numbers of samples. Dotblots are cumbersome to prepare and develop. The time required to wash between each step limits the number of tests that can be performed at one time while keeping incubation times constant. As a result, the dotblot should only be used to give a general estimate of reactivity for a small number of samples.

Using the ELISA as the "gold standard" the most appropriate method of interpreting dotblot results appears to be visual evaluation. This method is highly subjective and should only be used as a rough estimate of reactivity.

Western Blotting of Elk Serum Samples

Bands between 17 kDa and 70 kDa are most likely the result of binding of RB51 antigens with natural elk antibodies resulting from cross-reactive antigen exposures. These high molecular weight bands were rarely seen after development with the mouse monoclonal anti-bovine IgG_1 antisera and appeared only after re-development with the polyclonal anti-bovine antisera. These reactions could be due to inadequate blocking of the strips and resultant non-specific binding of non- IgG_1 antibodies to the transferred antigen. However, these bands consistently developed even when using numerous different blocking solutions suggesting that reaction with natural antibodies is the most plausible explanation.

The high molecular weight bands detected in all serum samples may account for the high amount of non-specific binding that was noted during elk ELISA development particularly with the

anti-IgG polyclonal reagent. Since the antibodies may have been produced in response to environmental antigens to which most wild-caught elk are exposed, diagnostic tests must be adequately specific to disregard these antibodies.

Bands between 6 and 17 kDa appear to react specifically with elk IgG_1 after RB51 vaccination. Antibodies to these RB51 antigens may be the ones being detected in the elk ELISA. However, no association was found between the number of bands within the 6 to 17 kDa range and the magnitude of percent positivity as determined by ELISA. Also, presence of these antibodies cannot be assumed to confer any level of protection against field strain isolates since passive transfer of anti-RB51 antibodies does not confer protection against challenge (6, 68, 79, 95). Since these are the only RB51 - specific bands noted with elk, future Western blot testing for evidence of RB51 vaccination should concentrate on these bands.

Chapter Three

Mice Inoculated with Elk RB51 Isolates

The experiments described in this chapter were performed to determine if RB51 isolates obtained from vaccinated elk underwent antigenic or microbiological alterations which may indicate that *in vivo* passage of the bacteria affects strain stability. For this, selected isolates were inoculated into mice and re-isolated from the mice at four weeks post-inoculation. The mice isolates were characterized biochemically and antigenically, examined for their virulence (by their ability to clear the spleen), and then compared to the original RB51 strain. Also, the humoral immune response of the mice to the inoculated isolates was determined by ELISA and Western blot analysis and compared to that of the original RB51 strain.

METHODS AND MATERIALS

Inoculation of Mice

Twenty-five 3.5 week old female BALB/c mice were purchased and allowed to acclimate to their environment for one week. They were divided into five groups of five mice each and ear notched for group identification.

Elk isolates chosen for mouse inoculation studies were identified as RB51 in the previous study, were all from bull elk (thereby eliminating gender bias), were separated temporally, and were recovered from more than one tissue source.

Table 3.1. Elk isolates chosen for mice inoculation

Elk #:	Experimental group:	Date isolated:	Isolate tissue of origin
20	Bull subcutaneously inoculated	05/23/95	blood
20	Bull subcutaneously inoculated	12/11/95	ampulla
36	Bull subcutaneously inoculated	05/23/95	blood

Each isolate to be inoculated was grown on two TSA plates for 48 hours, harvested with sterile TSB, and washed twice in sterile TSB. The bacteria were then diluted in sterile TSB to 4% transmittance at 525 nm, divided into 500 ul aliquots, and stored in multiple polypropylene microcentrifuge tubes at -70˚ C. The viable concentration of each isolate was determined by culturing ten fold serial dilutions of two randomly selected frozen microcentrifuge tubes. The appropriate dilution and volume of each isolate suspension to be injected was calculated to deliver 3 X 10^8 bacteria per mouse. The actual dose (listed in table 3.2) used to inoculate the mice was determined through serial dilutions immediately after each mouse group inoculation.

Table 3.2. Dose of isolates injected IP into each mouse group

Group #:	Isolate inoculated:	Actual dose (cfu) inoculated per mouse IP (x 10^8):
1	RB51	2.83
2	Biobullet RB51	3.19
3	Elk #20 Ampulla	2.34
4	Elk #20 Hemoculture	3.03
5	Elk #36 Hemoculture	2.82

Mice were bled from the retro-orbital plexus four days prior to and 2 weeks and 4 weeks post inoculation. All serum samples were stored in polypropylene microcentrifuge tubes at 4˚ C.

Commassie Blue Staining of SDS-PAGE

A gel containing 20 ul per antigen strip of each of the three elk isolates inoculated into the mice groups was electrophoresed as described under Antigen Preparation for SDS-PAGE and Western Blotting. The gel was stained by placing it in Commassie Blue R250 (0.1%) in water: methanol : glacial acetic acid (5:5:2 v/v) for two hours and then destained in distilled water : ethanol : glacial acetic acid (6.5 : 2.5 : 1 v/v) until bands could be easily visualized against a clear background (58). A digital scan and photograph of the gel were taken immediately after destaining.

Mouse Spleen Culture

Mice were euthanized by carbon dioxide inhalation at four weeks PI and their spleens aseptically removed. Each spleen was placed in a sterile polypropylene tube with one ml of sterile TSB and 0.75g of sterile (autoclaved) silica following established procedures (106). Briefly, spleens were ground with a glass rod. After allowing the silica to settle, all available fluid was aspirated from the tube and placed in a sterile polypropylene microfuge tube. Five, ten microliter drops of each sequential ten fold dilutions of the splenic suspension were plated on TSA plates and incubated in a humidified 37˚ C environment with 5% supplemental CO_2 for 5 days to allow maximal growth of colonies. The number of bacteria per spleen was then calculated based on colony forming units (cfu) observed per dilution.

Bacteriological and Biochemical Evaluation of Mice Isolates

Every colony recovered from each mouse spleen was numbered and subcultured on a TSA plate for further testing. Each colony was shown to lack an LPS related O-chain antigen by agglutination in 0.1% acriflavin solution (14), uptake of 0.05% crystal violet dye (133), and lack of color development in a BRU 38 colony blot test (101). After testing, all colonies from each mouse were combined to form one representative culture per mouse. These cultures were then further identified as *Brucella* bacteria based upon the results of the following tests:

- gram stain
- lysis in 3% KOH solution
- MacConkey agar
- Christensen's urease test media
- Simmon's Citrate test media
- oxidase test (Oxidase SpotTest - Difco - Detroit, Michigan)
- catalase test (3% H_2O_2)

- indole test (Indole SpotTest - Difco - Detroit, Michigan)

Mouse ELISA using RB51 Antigen

RB51 bacteria were grown and harvested as described in RB51 Antigen Preparation for Use in Elk ELISAs and Dotblots. Lyophilized acetone-killed RB51 microorganisms were reconstituted in sterile distilled water to 5% transmittance at 525 nm to make an antigen stock solution which was stored in polypropylene tubes at 4˚ C for no longer than one week. An antigen working solution was prepared immediately before use by centrifuging this stock solution at approximately 11,800g for 3 minutes, discarding the supernatant, and diluting the original volume of killed RB51 1:20 v/v in bicarbonate phosphate buffer, pH 9.6. Appropriate wells of a 96-well medium-binding polystyrene plate (Costar - Kennebunk, ME) were coated with 200 ul of RB51 working solution, sealed with parafilm sheet, and incubated in a humidified chamber at 4˚ C overnight.

The plates were allowed to equilibrate to room temperature and were washed four times with freshly prepared phosphate buffer solution containing 0.05% Tween 20 (PBST-20) using a semi-automated plate washer (NUNC Immunowash - Naperville, IL). For each washing, wells were filled with approximately 250 ul of PBST-20, allowed to soak for approximately 20 seconds, and then emptied by suction. After the fourth washing, the plates were inverted and tapped on a countertop to fully empty the wells. However, the wells were never allowed to completely dry as this can cause high non-specific binding (89). No blocking step was used as all tested blocking solutions unexpectantly caused high non-specific background. Serum samples were diluted 1:50 and 1:100 in PBST-20 in a 96-well polypropylene plate (Costar - Kennebunk, ME) immediately before dispensing. Two hundred microliters of each diluted serum sample was incubated in the original antigen coated polystyrene plate at 37˚ C for 30 minutes. After incubation, the wells were washed four times. Each well was then incubated at 37˚ C with 200 ul of a polyclonal peroxidase-conjugated goat IgG fraction to mouse IgG whole molecule (Cappel - West Chester, PA) diluted 1:800 in PBST-20. Next, the plates were washed four times. A developing solution was prepared immediately before use by mixing 10 mg of ortho-phenylenediamine (Sigma - St. Louis, MO) in 1 ml of 100% methanol with 100 ml distilled water and 100 ul of 30% H_2O_2. The wells were then incubated with 200 ul of developing solution at room temperature on a shaking platform (Baxter Scientific) at 120 RPM for 30 minutes. The developing reaction in each well was stopped with 40 ul of 0.18M H_2SO_4. The optical density of each well at 490 nm was determined by a 96-well plate reader (Molecular Devices).

Mouse ELISA Controls

All serum sample dilutions were tested in duplicate wells to help assess inter-well variability. Positive and negative control wells were tested in each plate. Positive control wells were incubated with pooled mouse serum which contained a high level of anti- RB51 antibodies obtained from previous experiments. Negative control wells consisted of the following:

- Pre-inoculation serum samples: two serum samples obtained from each mouse prior to vaccination with RB51 or saline.
- 'No antigen' wells: two wells for each serum sample diluted 1:50 which were not coated with the RB51 antigen stock solution. Instead, these wells were incubated overnight with 200 ul of the bicarbonate phosphate buffer only.
- 'No serum' wells: two wells containing all appropriate reagents except serum. Two hundred microliters of PBST-20 were used instead of the diluted serum.

Wells containing all reagents except conjugated antisera were not routinely included since previous work indicated that the conjugated antisera did not bind nonspecifically and thus, did not cause background reading problems.

Mouse ELISA using Selected Elk and Biobullet Derived Isolates as Antigen

Each selected elk isolate to be used as an antigen in the ELISA was prepared as described in Mouse ELISA using RB51 Antigen. Briefly, each isolate was plated on two TSA plates and incubated at 37˚ C and 5% CO_2 for 72 hours. The bacteria were harvested with 3-4 ml of sterile distilled water and killed by stirring with an equal volume of acetone for three hours. After washing twice with sterile distilled water, the organisms were frozen to -70˚ C and lyophilized. ELISA plates were coated with antigen as described in Mouse ELISA Using RB51 Antigen.

Mouse sera were pooled from each inoculation group (RB51, Biobullet RB51, Elk #20 Ampulla, Elk #20 Hemoculture, and Elk #36 Hemoculture - see table 3.2) by combining equal volumes of serum from each bleeding (prevaccination, 2 weeks PI, and 4 weeks PI) from all mice within a group. Serums were stored at 4˚ C in polypropylene tubes. Two hundred microliters of each serum diluted 1:50 and 1:100 in PBST-20 were tested in wells as described in Mouse ELISA using RB51 Antigen. Controls were used as described in Mouse ELISA Controls.

SDS - PAGE and Western Blot Analysis of Mouse Samples

Antigens for electrophoresis were prepared as described in Antigen Preparation for Gel Electrophoresis and Western Blotting. SDS-PAGE was performed as described in SDS - PAGE using both RB51 and the three selected Elk isolates (Elk #20 Ampulla, Elk #20 Hemoculture, and Elk #36 Hemoculture) as antigens.

After transfer, the nitrocellulose sheet was agitated with 0.25% gelatin (Difco - Detroit, MI) in TBS for one hour to block non-specific binding of antibodies. Serum samples from each bleeding (Pre-inoculation, 2 weeks PI, and 4 weeks PI) within each group were pooled and tested against both RB51 and the isolate with which the group was inoculated. The sheet was then cut into strips corresponding to transferred antigen columns and agitated overnight in pooled mouse serum diluted 1:50 in TBS. All samples from each mouse group were tested on the same transfer to facilitate comparisons between different bleeds of the same animal group. Each strip was then washed five times in TBST for two minutes and then agitated with a 1:500 dilution of peroxidase-conjugated goat anti-mouse IgG (whole molecule) (Cappel -- West Chester, PA) in TBS for one hour. The strips were washed again in TBST and then twice in distilled water to remove any residual Tween which might cause non-specific color development. The developing solution was prepared by mixing 60 mg of 4-chloro-1-naphthol (Sigma - St. Louis, MO) dissolved in 10 ml methanol with 100 ml of TBS and 600 ul of 30% H_2O_2. After washing, the nitrocellulose was agitated in the developing solution until positive control strips showed color development but before the entire strip turned uniformly purple. The nitrocellulose was briefly rinsed in distilled water and allowed to dry in a cool, dark area. A photograph and a digital scan was made of each strip within twelve hours.

Controls used for mouse Western blotting were those described under Controls used in Gel Electrophoresis and Western Blotting of Mouse Serum Samples

Mouse Isotype and Sub-isotype Determination by Western Blot Analysis

RB51 antigen for electrophoresis was prepared as described in Antigen Preparation for Gel Electrophoresis and Western Blotting. SDS-PAGE was performed as described in SDS -PAGE. Except for the molecular weight marker strip, all strips were run with 20 ul of RB51 antigen diluted with sample buffer solution.

After transfer the nitrocellulose sheet was agitated with 0.25% gelatin (Difco - Detroit, MI) in TBS for one hour to block non-specific binding to the nitrocellulose. Serum samples from each bleeding (Pre-inoculation, 2 weeks PI, and 4 weeks PI) of the RB51 mouse group were pooled by bleed. One nitrocellulose sheet was agitated overnight with one of the three pooled mouse serums diluted 1:50 in TBS. All nitrocellulose strips incubated with each bleed were cut from the same nitrocellulose transfer sheet to facilitate comparisons among different anti-isotypes. Each strip was then washed five times in TBST for two minutes. Next, they were agitated with a 1:1000 dilution of one of the following five peroxidase-conjugated monoclonal goat anti-mouse isotypes (Cappel - West Chester, PA)**:**

IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM

or with a 1:500 dilution of goat polyclonal anti-mouse IgG whole molecule (Cappel - West Chester, PA) for one hour. The strips were washed again in TBST and then twice in distilled water to remove any residual Tween which might cause non-specific color development (89). The developing solution was prepared by mixing 60 mg of 4-chloro-1-naphthol (Sigma - St. Louis, MO) dissolved in 10 ml methanol with 100 ml of TBS and 600 ul of 30% H_2O_2. After washing, the nitrocellulose was agitated in the developing solution until positive control strips showed color development but before the entire strip turned uniformly purple. The nitrocellulose was briefly rinsed in distilled water and allowed to dry in a cool, dark area. A photograph and a digital scan were made of each strip within twelve hours. The strips were then evaluated for color development specific for the post-inoculation serum samples which would indicate specific isotype response to RB51 antigens.

Identical controls were used in this test as described in Controls used in Gel Electrophoresis and Western Blotting of Mouse Serum Samples.

Western Blot to Prove the Absence of Anti-O-Chain Antibody Production in Mice Inoculated with RB51 and Elk Isolates

SDS-PAGE and Western blotting were performed as described under SDS - PAGE and Western Blotting of Mouse Serum Samples. However, each gel was electrophoresed with seven strips of 15 ul of *B. abortus* strain 2308 (prepared as described in Antigen Preparation for Gel Electrophoresis and Western Blotting) and one strip of 20 ul of RB51 and then transferred onto nitrocellulose sheets. The 2308 bound strips cut from the same nitrocellulose sheet were incubated with a 1:50 dilution in TBS of one of the following four serum samples:

- ◊ a pooled two week post-inoculation serum sample from each mouse group.
- ◊ a pooled four week post-inoculation serum sample from each mouse group.
- ◊ serum from a 2308 infected mouse.
- ◊ monoclonal BRU 38.

The one RB51 nitrocellulose strip from each gel was incubated with a 1:50 dilution in TBS of serum from a 2308 infected mouse.

The strip incubated with BRU 38 was agitated with a 1:500 dilution of peroxidase-conjugated polyclonal goat anti-rat IgG (whole molecule) (Cappel - West Chester, PA) for one hour. All other strips were incubated in a 1:500 dilution of peroxidase-conjugated polyclonal goat IgG fraction to mouse IgG whole molecule (Cappel - West Chester, PA) for one hour. After development and drying, the strips were examined for the banding pattern characteristic of the LPS associated O-chain of smooth *Brucella spp.* (106).

RESULTS

Commassie Blue Staining of SDS-PAGE

Below is a link to the digital image of the Commassie Blue stained SDS-PAGE of RB51, Biobullet- derived RB51, and all elk-derived isolates used to inoculate mice groups. The organism electrophoresed in each lane is indicated.

Figure 3.1. SDS-PAGE of selected elk isolates, RB51, Biobullet, and molecular weight standard.

No differences were noted between the banding patterns of the RB51 organism and the elk or biobullet derived isolates.

Mouse Spleen Culture

Table 3.3 lists the colony forming units isolated from the spleens of each mouse euthanized at 4 weeks PI.

Table 3.3. Total colony forming units (cfu) isolated from each mouse spleen 4 weeks PI

Group #:	Isolate inoculated:	cfu Isolated per mouse spleen	Average log cfu/group
1	RB51	60 40 40 0 20	1.2566
2	Biobullet RB51	0 0 60 280 20	1.1052
3	Elk #20 Ampulla	20 40 40 20 20	1.4214
4	Elk #20 Hemoculture	20 40 40 100 20	1.5612
5	Elk #36 Hemoculture	140 1380 80 320 620	2.4972

The log of the number of colony forming units (cfu) per spleen for every mouse was computed and the resultant values were found to be normally distributed. Differences between the log of the cfu's obtained from each group were tested using Bonferroni pairwise comparisons of means ($p < 0.050$ - Statix version 4.0 analytic software - Tallahassee, FL) to determine if any isolate inoculation was associated with a statistically significant higher recovery of cfu's. Since the RB51 inoculated group (group 1) was the control group, any group which had a significantly different number of cfu's would suggest a change in virulence. Evaluation of the groups showed that no group was statistically distinct from the group inoculated with the original RB51 subcutaneous vaccine based on cfu's recovered. The number of cfu's recovered from mice inoculated subcutaneously with the elk #36 hemoculture isolate (group 5) was shown to be distinct from the mice group inoculated with the biobullet derived RB51.

Bacteriological and Biochemical Evaluation of Mice Isolates

All mice isolates were shown to lack an LPS related O-chain by agglutination in 0.1% acriflavin solution, uptake of 0.05% crystal violet dye, and lack of color development in a BRU 38

colony blot test. Table 3.4 illustrates the results of bacteriological and biochemical tests used to further classify mice isolates as *Brucella* bacteria.

Table 3.4. Bacteriological and biochemical evaluation of mice isolates

Mouse Group*	**Gram Stain**	**Lysis in 3% KOH**	**MacConkey agar**	**Christensen's urease test media**	**Simmon's citrate test media**	**Oxidase SpotTest**	**Indole SpotTest**	**Catalse test**
RB51	-	+	-	+	-	+	-	+
Biobullet RB51	-	+	-	+	-	+	-	+
Elk #20 Ampulla	-	+	-	+	-	+	-	+
Elk #20 Hemoculture	-	+	-	+	-	+	-	+
Elk #36 Hemoculture	-	+	-	+	-	+	-	+

*all mice produced identical results within each group

Based on these results, every isolate cultured from each mouse's spleen lacked an 0-chain. When all isolates from each mouse were combined to form one representative isolate per mouse, each isolate was shown to belong to the *Brucella* genus.

Mouse ELISA using RB51 Antigen

Percent positivity of the mice serums were calculated as discussed in Chapter Two with a few exceptions. The serum used as a positive control was a pool of samples recovered from a group of mice previously inoculated with RB51. Since mice within each group were not individually marked, successive serum samples from each individual animal could not be identified. Therefore, the average O.D. value for all pre-inoculation mouse serum samples within each group was calculated and used in the calculation of percent positivity for each post-inoculation sample. Since each mouse was not individually identified, the progression of percent positivity from two to four weeks PI for each mouse could not be followed. Therefore, the percent positivity values from each group were evaluated by a one way analysis of variance using a statistical program (Statix version 4.0 Tallahassee, FL) to determine if two and four week PI values significantly varied between group.

Table 3.5. O.D. values of sera (diluted 1:100) from inoculated mice in ELISA using whole cell RB51 antigen at pre-inoculation and 2 and 4 weeks post-inoculation

Group	Pre*	Pre	Pre	Pre	Pre	Average all Pre	+ control	no serum
RB51	0.120	0.209	0.083	0.082	0.092	0.117	1.720	0.055
Biobullet	0.120	0.161	0.124	0.145	0.130	0.136	1.846	0.050
20 Ampulla	0.115	0.166	0.138	0.127	0.116	0.132	1.840	0.058
20 Hemoculture	0.129	0.124	0.094	0.175	0.130	0.130	1.960	0.054
36 Hemoculture	0.130	0.140	0.106	0.114	0.082	0.114	1.962	0.060

*Pre-inoculation serum samples

Group	2 wks*	2 wks	2 wks	2 wks	2 wks
RB51	0.524	0.704	0.816	1.030	0.816
Biobullet	1.034	0.794	0.218	1.144	0.824
20 Ampulla	0.594	0.745	0.542	0.592	0.762
20 Hemoculture	0.973	0.616	0.784	1.108	0.928
36 Hemoculture	0.883	0.862	0.894	0.704	1.178

*serum samples from two weeks post-inoculation

Group	4 wks*	4 wks	4 wks	4 wks	4 wks
RB51	1.331	1.608	1.504	1.004	0.894
Biobullet	1.174	1.675	1.257	0.982	1.647
20 Ampulla	1.087	0.912	1.182	1.610	0.811
20 Hemoculture	1.112	1.703	2.162	1.122	1.455
36 Hemoculture	1.408	1.429	1.452	1.722	1.425

*serum samples from four weeks post-inoculation

Table 3.6. Percent positivity values of sera (diluted 1:100) from mice in ELISA using whole cell RB51 antigen at pre-inoculation and 2 and 4 weeks post-inoculation

Group	2 Wks*	2 Wks	2 Wks	2 Wks	2 Wks	Average	SD^
RB51	24.4	35.3	42.0	54.8	42.0	39.7	11.1
Biobullet	50.0	36.6	4.6	56.1	38.3	37.1	19.9
20 Ampulla	25.9	34.4	23.0	25.8	35.4	28.9	5.6
20 Hemoculture	44.2	25.5	34.3	51.3	41.9	39.4	9.9
36 Hemoculture	40.4	39.3	41.0	31.0	55.9	41.5	9.0

*serum samples from two weeks post-inoculation
^one standard deviation

Group	4 Wks*	4 Wks	4 Wks	4 Wks	4 Wks	Average	SD^
RB51	72.9	89.5	83.3	53.3	46.7	69.1	18.6
Biobullet	57.8	85.7	62.4	47.1	84.1	67.4	16.9
20 Ampulla	53.6	43.8	58.9	82.9	38.1	55.5	17.4
20 Hemoculture	51.5	82.5	106.6	52.0	69.5	72.4	23.1
36 Hemoculture	68.0	69.1	70.3	84.5	68.9	72.2	17.4

*serum samples from four weeks post-inoculation
^one standard deviation

Statistical analysis revealed that the anti-RB51 antibody response detected at two and four weeks PI did not significantly vary among mouse inoculation groups ($p < 0.05$).

Mice ELISA using Elk and Biobullet Derived Isolates as Antigen

Percent positivity values were computed as discussed in Mice ELISA using RB51 as Antigen and were compared in a t-test (Statix version 4.0 Tallahassee, FL) between ELISAs using RB51 or the specific isolate as the antigen.

The following data (Table 3.7) represents the optical density values obtained from ELISAs in which serum from each mouse group was reacted using its original inoculum as antigen in the ELISA instead of the original strain RB51.

Table 3.7. O.D. values of sera (diluted 1:100) from inoculated mice in ELISA using the respective original mouse inoculums as antigen

ß	Pre	Pre	Pre	Pre	Pre	Average of all Pre	+ control	no serum
Biobullet *	0.088	0.089	0.076	0.104	0.092	0.090	1.248	0.044
20 Ampulla+	0.114	0.117	0.080	0.112	0.119	0.108	1.500	0.050
20 Hemoculture!	0.103	0.087	0.086	0.089	0.073	0.088	1.292	0.048
36 Hemoculture#	0.137	0.149	0.109	0.187	0.108	0.138	1.672	0.079

***** biobullet derived RB51 organisms used as ELISA antigen
+ Elk #20 ampulla isolate used as ELISA antigen
! Elk #20 hemoculture isolate used as ELISA antigen
Elk #36 hemoculture isolate used as ELISA antigen

Group	2 Wks PI	2 Wks PI	2 Wks PI	2 Wks PI	2 Wks PI
Biobullet*	0.524	0.442	0.114	0.412	0.499
20 Ampulla+	0.516	0.485	0.386	0.367	0.680
20 Hemoculture!	0.404	0.398	0.349	0.390	0.448
36 Hemoculture#	0.780	0.750	0.870	0.870	0.947

***** biobullet derived RB51 organisms used as ELISA antigen
+ Elk #20 ampulla isolate used as ELISA antigen
! Elk #20 hemoculture isolate used as ELISA antigen
Elk #36 hemoculture isolate used as ELISA antigen

Group	4 Wks PI	4 Wks PI	4 Wks PI	4 Wks PI	4 Wks PI
Biobullet*	0.664	0.802	0.670	0.518	1.028
20 Ampulla+	0.822	0.690	0.784	1.014	0.540
20 Hemoculture!	0.538	0.806	1.146	0.526	0.520
36 Hemoculture#	0.937	1.093	1.070	1.067	0.993

***** biobullet derived RB51 organisms used as ELISA antigen
+ Elk #20 ampulla isolate used as ELISA antigen
! Elk #20 hemoculture isolate used as ELISA antigen
Elk #36 hemoculture isolate used as ELISA antigen

Table 3.8 represent the percent positivity values obtained using the above tabulated optical density values

Table 3.8. Percent positivity values of sera (diluted 1:100) from mice in ELISA using elk isolates and biobullet as antigen.

Group	2 Wks PI	2 Wks PI	2 Wks PI	2 Wks PI	2 Wks PI	Average	SD^
Biobullet *	36.0	29.2	2.0	26.7	34.0	25.6	13.7
20 Ampulla+	28.1	26.0	19.2	17.9	39.4	26.1	8.6
20 Hemoculture!	25.4	24.9	21.0	24.3	28.9	24.9	2.8
36 Hemoculture#	40.3	38.4	46.0	46.0	50.8	44.3	5.0

***** biobullet derived RB51 organisms used as ELISA antigen
+ Elk #20 ampulla isolate used as ELISA antigen
! Elk #20 hemoculture isolate used as ELISA antigen
Elk #36 hemoculture isolate used as ELISA antigen
^ one standard deviation

Group	4 Wks PI	4 Wks PI	4 Wks PI	4 Wks PI	4 Wks PI	Average	SD^
Biobullet *	47.7	59.1	48.2	35.5	77.9	53.7	15.9
20 Ampulla+	49.2	40.1	46.6	62.5	29.8	45.6	12.0
20 Hemoculture!	36.2	57.7	85.0	35.2	34.7	49.8	22.0
36 Hemoculture#	50.2	59.9	58.5	58.3	53.7	56.1	4.0

***** biobullet derived RB51 organisms used as ELISA antigen
+ Elk #20 ampulla isolate used as ELISA antigen
! Elk #20 hemoculture isolate used as ELISA antigen
Elk #36 hemoculture isolate used as ELISA antigen
^ one standard deviation

Statisitcal analysis (t-test, $p<0.05$) indicated that regardless of the antigen source for the ELISA, mice had similar antibody responses.

SDS - PAGE and Western Blot Analysis of Mouse Samples

Below are links to images of Western blots in which each mouse group pooled serum (Pre-inoculation, 2 weeks PI, and 4 wks PI) was reacted against either RB51 or the isolate used to inoculate each group. Each image is labeled with the antigen(s) and mouse serum sample tested.

Figure 3.2. Western blot with pooled sera (pre-inoculation, 2 weeks post-inoculation, and 4 weeks post-inoculation) from the RB51 mouse group reacted against whole cell RB51.

Figure 3.3. Western blot with pooled sera (pre-inoculation, 2 weeks post-inoculation, and 4 weeks post-inoculation) from the Biobullet mouse group reacted against whole cell RB51 and Biobullet isolate antigens.

Figure 3.4. Western blot with pooled sera (pre-inoculation, 2 weeks post-inoculation, and 4 weeks post-inoculation) from the elk #20 ampulla mouse group reacted against whole cell RB51 and elk #20 ampulla isolate antigens.

Figure 3.5. Western blot with pooled sera (pre-inoculation, 2 weeks post-inoculation, and 4 weeks post-inoculation) from the elk #20 hemoculture mouse group reacted against whole cell RB51 and elk #20 hemoculture isolate antigens.

Figure 3.6. Western blot with pooled sera (pre-inoculation, 2 weeks post-inoculation, and 4 weeks post-inoculation) from the elk #36 hemoculture mouse group reacted against whole cell RB51 and elk #36 hemoculture isolate antigens.

Western blot analysis indicated that within each mouse group, identical banding patterns developed when reacting each mouse group's sera against either RB51 or the isolate used to inoculate each group. Differences among groups were observed within the bands ranging from 17 to 60 kDa.

Table 3.9 lists the calculated molecular weights (MW) for each observed band within the 17–60 kDa range. The number of bands outside of this range are indicated. Since the same bands were found with both RB51 and RB51 isolates within each group, the molecular weights listed represent bands developed against both antigens.

Table 3.9. Molecular weight (kDa) of bands observed in Western blot analysis using mouse sera versus RB51 and respective inoculum antigens.

Mouse group:	RB51 inoculated	Biobullet inoculated	Elk #20 Ampulla inoculated	Elk #20 Hemoculture inoculated	Elk #36 Hemoculture inoculated
number of bands with MW between 60 and 148 kDa	3	3	2	2	3
number of bands with MW between 17 and 60 kDa	4[a]	3[b]	4[c]	4[d]	3[e]
number of bands with MW < 17 kDa	1	1	1	1	1

a. molecular weights = 56, 49, 19, and 16
b. molecular weights = 54, 20, and 17
c. molecular weights = 54, 48, 20, and 17
d. molecular weights = 58, 51, 19, and 16
e. molecular weights = 53, 19, and 17

In all groups, two to three bands were revealed with post-inoculation sera which have molecular weights between 60 and 148 kDa and one band was consistently noted below 17 kDa. The molecular weights of these cannot be calculated as they are outside of the linear range of a

12.5% polyacrylamide gel. All groups produced three to four bands with molecular weights of approximately 55, 49, 19, and 17 kDa.

Mouse Isotype and Sub-isotype Determination by Western Blot Analysis

Below are links to images of Western blots which depict the reaction of RB51 antigen versus pooled serum from the RB51 inoculated mouse group (pre-inoculation, 2 weeks PI, or 4 weeks PI) which were developed with six different anti-(sub)isotype-antibodies.

Figure 3.7. Western blot of RB51 vaccinated mouse serum obtained prior to vaccination reacted with RB51 antigen and developed with different (sub)isotype anti-sera.

Figure 3.8. Western blot of RB51 vaccinated mouse serum obtained 2 wks PI reacted with RB51 antigen and developed with different (sub)isotype anti-sera.

Figure 3.9. Western blot of RB51 vaccinated mouse serum obtained 4 wks PI reacted with RB51 antigen and developed with different (sub)isotype anti-sera

Using original RB51 as the antigen source, a small amount of non-specific binding was noted with the serum samples obtained prior to inoculation which were incubated with monoclonal anti-mouse IgG2a. Multiple bands were noted with post-vaccination serum samples developed with monoclonal anti-mouse IgG2a, IgG2b, IgG3, and the polyclonal anti-mouse antisera. The molecular weight of each band observed with the different mouse sub-isotypes is summarized in Table 3.10.

Table 3.10. Number or molecular weight (kDa) of bands observed in Western blot analysis using serum of RB51 inoculated mouse group at 2 or 4 wks PI versus RB51 antigen developed with six different anti-(sub)isotypes.

Conjugate antisera:	IgG_1		IgG_{2a}		IgG_{2b}		IgG_3		IgM		Polyclonal IgG	
	2 Wks PI	4 Wks PI	2 Wks PI	4 Wks PI	2 Wks PI	4 Wks PI	2 Wks PI	4 Wks PI	2 Wks PI	4 Wks PI	2 Wks PI	4 Wks PI
number of bands between 60 and 148 kDa	-	1	3^1	4^2	3^1	4^2	3^1	4^2	-	-	-	2
MW (kDa) of bands between 60 and 17 kDa	-	-	21	54	57	57	57	57	-	-	22	57
				21		51						21
				18		19						
number of bands with MW < 17 kDa	-	-	-	1^3	-	1^3	-	-	-	-	-	1^3

superscript numbers indicate groups of bands observed on the same nitrocellulose sheet that appear to be identical based on position.

Only one band with MW between 60 and 148 kDa developed at four weeks with post-inoculation sera developed with anti-IgG_1. No bands developed on the strips incubated with anti-IgM antisera.

Western Blot to Prove the Absence of Anti-O-Chain Antibody Production in Mice Inoculated with RB51 and Elk Isolates

Below are links to digital images which illustrate the serological response of mice inoculated with strain RB51 (at 2 or 4 weeks PI) or strain 2308 reacted against whole cell strain 2308 antigen. Monoclonal BRU 38 (a monoclonal antibody specific for the LPS associated O-chain of smooth *Brucella spp.*) was also reacted against both whole cell strain 2308 antigen and to whole cell RB51 antigen.

Figure 3.10. Pooled sera obtained (two weeks post-inoculation) from each mouse experimental group reacted with whole cell *B. abortus* strain 2308 and developed with horseradish peroxidase conjugated polyclonal IgG anti-mouse.

Figure 3.11. Pooled sera obtained (four weeks post-inoculation) from each mouse experimental group reacted with whole cell *B. abortus* strain 2308 and developed with horseradish peroxidase conjugated polyclonal IgG anti-mouse.

As expected, the characteristic O-chain associated smearing pattern above 22 kDa of smooth *Brucella spp.* appeared with the 2308 nitrocellulose strips incubated with serum from a 2308 infected mouse. Serum from this same 2308 infected mouse incubated with a stock culture of RB51 bound strip did not produce this smear and produced only two bands at approximately 57 and 61 kDa. The 2308 antigen strips incubated with serum samples from RB51 infected mouse groups at 2 and 4 weeks PI did not show the LPS associated O-chain smear and consistently produced ten bands: four bands between 60 and 148 kDa, a band at 56 kDa, 53 kDa, 22 kDa, and 20 kDa respectively, and two bands below 17 kDa illustrating the broad antigen cross-reactivity (with the exception of O-chain) between strain 2308 and RB51. The specificity of the "smear" was confirmed by the use of BRU 38 reacted against whole cell strain 2308.

BRU 38 Colony Blot Test

After testing every isolate cultured from each mouse, all but one isolate (colony #25 from one mouse inoculated with the isolate from elk # 36 Hemoculture) were shown to be rough (non-reactive). A small amount of color development was observed on the nitrocellulose sheet close to colony #25. The colony blot was repeated using only colony #25 from elk #36 and appropriate controls which showed that the isolate was indeed rough.

DISCUSSION

Commassie Blue Staining of SDS-PAGE

RB51, biobullet RB51, and the three elk isolates (elk # 20 Ampulla, elk #20 Hemoculture, and elk #36 Hemoculture) showed no visible differences after SDS-PAGE and Commassie Blue staining. This suggests that the protein profile of RB51 remains unchanged after passage through elk. However, small structural changes would not likely be noticed with this test. Also changes in

the secondary or tertiary configuration of the proteins could not be detected since the proteins were denatured with SDS before electrophoresis.

Mouse Spleen Culture

Statistical analysis of the number of cfu recovered from spleens at four weeks post-inoculation found that groups of mice inoculated with either an elk-derived isolate or RB51 Biobullet did not differ from the RB51 inoculated group (control group). Since the persistence of organisms in mice spleen can be an indication of a *Brucella 's* virulence (106), none of the inoculated bacteria appear to have altered their virulence significantly from the original RB51. As a result, it can be concluded that the virulence of RB51 was not appreciably altered by passage through elk. Also, the processing of the RB51 organisms into lactose based pellets did not cause detectable alterations in the organisms' virulence as assessed by the mouse clearance model.

Since the number of cfu's cultured from mouse group 5 (inoculated with elk #36 hemoculture) and group 2 (inoculated with biobullet RB51) were shown to be dissimilar by Bonferroni pairwise comparisons of means ($p < 0.05$) slight changes could have occurred in one or both of these organisms. This difference could be due to a slight decrease in the virulence of the biobullet derived RB51 organisms due to processing (which included a passage on potato agar) and/or a slight increase in the virulence of the isolate from elk #36 hemoculture. It appears unlikely in light of the behavior of the "original" RB51 and the other isolates that passage in elk did increase the virulence of RB51. It is possible that the differences noted in cfu between these two groups may be due to variability experienced at both inoculation of mice and in recovery of isolates. Future research should include a confirmation of these results and a characterization of the organism's DNA to note any alterations from the original strain of RB51.

Bacteriological and Biochemical Evaluation of Mice Isolates

Bacteriological and biochemical tests indicate that all isolates recovered from RB51 infected mice belong to the *Brucella* genus. The lack of the LPS-associated O-side chain in the mice isolates demonstrate that the elk isolates remained stable through elk and mouse passage and therefore had not reverted to a smooth strain of *Brucella*. The stability of RB51 demonstrated in this experiment is vital for the organism's use as a serologically distinct, attenuated vaccine that will not induce anti - O antibodies.

Mouse ELISA using RB51 Antigen

Mouse sera were tested by ELISA to determine the relative level of anti-RB51 antibodies. The objective of the study was to determine if mice inoculated with elk-derived RB51 isolates or biobullet isolates would produce varying antibody levels against RB51 as determined by ELISA.

Statistical analysis (by a one way analysis of variance using Statix version 4.0 Tallahassee, FL) revealed that the detectable anti-RB51 antibody response observed at two and four weeks PI did not significantly vary by group ($p < 0.05$). This would suggest that the antigenic profile of each isolate responsible for the induction of antibodies detectable in the ELISA is similar to that of the original culture of RB51 and is not significantly affected by passage through elk or processing into biobullets.

Mice ELISA using Elk and Biobullet Derived Isolates as Antigen

Mouse sera were tested in an ELISA using the isolate inoculated in each group as the ELISA antigen. The objective of this test was to determine if passage through elk or processing into biobullets would cause an alteration in the antigenic profile of the RB51 organism which would result in a modified antibody response detectable by ELISA. Percent positivity values for each bleeding within a mouse group were compared with ELISAs using RB51 antigen and elk or biobullet derived isolate antigens. Different percent positivity values for the same bleed within a group would suggest antigenic alteration of the elk or biobullet derived isolate.

Statistical analysis revealed no differences when testing samples against RB51 or the original inoculum. This would further support the hypothesis that RB51 did not alter antigenically by passage through elk or by processing into biobullets.

SDS-PAGE and Western Blot Analysis of Mouse Samples

The similar banding pattern seen among the five mice groups indicate that regardless of the RB51 inoculum, the mice produce anti-RB51 antibodies specific for the same antigens of RB51. Small antigenic variations occurring at single epitopes can not be detected by this method since the antigens were denatured in SDS prior to electrophoresis.

Variation in the number of antigen bands recognized by serum from each group could be the result of a slightly lower antibody response by either the entire group or (an) individual(s) thereby falling below the detection level of the Western blot.

The similar Western blot results between the mice groups indicate that the antigenic profile of *B. abortus* strain RB51 as detected in Western blot is stable after passage through elk and then mice. This would indicate that the RB51 vaccine derived from a low passage number of a master seed should be stable and not give rise to variants in the field.

Two clear, completely white bands of greater than 60 kDa were also noted on strips that were slightly over-developed (had a uniformly purplish background). These clear bands appeared to repel some or all reagents such that a well-defined white band appeared against the strip's slightly dark background. These bands may represent hydrophobic areas of the RB51 antigen. The bands may represent the hydrophobic phospholipid regions (99) of the bacteria's cell membrane yet further study would be needed to accurately identify the cause of these clear bands.

Mouse Isotype and Sub-isotype Determination by Western Blot

As seen in this experiment, IgG2a, IgG2b, and IgG3 isotype antibodies are produced against many different antigens in RB51 and are likely the predominate antibody response to RB51 infection. This response reaches detectable levels (in Western blot) by two weeks post vaccination and continues at least until four weeks PI. Mice IgG_1 isotype antibodies are produced against only one RB51 protein. As no bands were noted in strips incubated with anti-IgM antisera, IgM antibodies against RB51 antigens do not seem to be detected in this test. This observation may be due to problems with the specific anti-IgM antisera since no positive controls were used to insure the quality of this reagent. Overall, it appears that several RB51 antigens elicit an antibody response of multiple mice isotypes.

Presumably, one or many of the bands recognized by the polyclonal IgG antisera is(are) responsible for the antibody response detected in the mouse ELISA system. The specific bands associated with reactivity in the ELISA were not identified but they may be identified by absorption

experiments. The mouse sera could be incubated with the whole cell RB51 used as the ELISA antigen, the mixture centrifuged, and the resultant absorbed serum supernatant incubated with RB51 bound nitrocellulose strips. After development with polyclonal anti-mouse antisera, the strip could be compared to an identical strip incubated with non-absorbed mouse sera. The band(s) most likely responsible for the antibody response in the ELISA may not be visible on the strips reacted with the absorbed serum.

Western Blot to Prove the Absence of Anti-O-Chain Antibody Production in Mice Inoculated with RB51 and Elk Isolates

Western blot strips of strain 2308 whole cell antigen incubated with 2308 infected mouse sera and with BRU 38 (a monoclonal antibody specific for the LPS associated O-chain of smooth *Brucella spp.*) revealed a characteristic "smear" (106) and demonstrated the presence of antibodies to the O-chain of the LPS component of smooth Brucella spp. The lack of the LPS smear with 2308 antigen strips incubated with serum from RB51 infected mice indicated the absence of O-specific antibodies in these sera and served as a negative control for the detection of LPS in serum samples. Nevertheless pooled serum samples from all mouse groups at both two and four weeks post-inoculation revealed multiple bands clearly indicating the cross-reactivity among strain 2308 and RB51 non-O-chain antigens.

Lack of anti-O side-chain antibodies in the experimental mice groups further supports the bacteriological observations that the RB51 organisms have not reverted to smooth organisms. A reversion would interfere in serological diagnosis of RB51 vaccinated animals, could increase the virulence of RB51, and could cause disease in the vaccinated animals.

BRU 38 Colony Blot Test

All isolates tested negative indicating that isolates had not reverted. Although one isolate did test positive (colony #25). Retesting of the original culture of isolate #25 obtained from a mouse inoculated with an isolate from elk #36 hemoculture indicated the organism's rough characteristics, the original color development was attributed to laboratory error. This error could be the result of contamination of the isolate during culture and transfer. Also, *B. abortus* 2308 organisms transferred onto the nitrocellulose sheet could have smeared onto the area of the isolate during incubation, bound to the nitrocellulose, and caused color development. This latter situation occurred in another colony blot in which a heavy culture of 2308 'smeared' onto an area with no other isolate and resulted in a blur of color development. The color development could not be due to a mixed culture of rough and smooth organisms as each colony is composed of the progeny of a single bacteria.

Since all mice isolates were rough by the BRU 38 colony blot, the elk isolates inoculated into the mice appear to be stable during passage in mice. This fact supports previous research that has shown RB51 to be stable during multiple animal passages (106).

Chapter Four

A Brief Review of the Humoral Immune Response in RB51 Vaccinated Elk and Mice

Vaccine *Brucella abortus* strain RB51, unlike the wild strain 2308 and another vaccine strain (strain 19) does not induce anti-O-chain antibodies. An efficacious vaccine strain that fails to produce an O-chain and thus a lack of an anti-O-chain humoral response greatly simplifies identification of vaccinated versus field strain infected animals. Although strain RB51 has been shown to remain rough in passage through mice and cattle, only preliminary studies have been performed to assess its stability in elk. Nearly 100% stability in elk would be required for RB51 to be considered as a useful vaccine strain in this species.

The three primary objectives of this research were the following: 1) to develop a serological assay to detect anti-RB51 antibodies in vaccinated elk (*Cervus elaphus nelsoni)*, 2) to identify potential antigenic alterations in RB51 after vaccination of elk and BALB/c mice, and 3) to confirm the general stability of RB51.

Sixty-four elk were divided into four groups based on the route of inoculation of viable bacteria. One bull elk group and one female elk group were subcutaneously inoculated with bacteria, one female elk group was ballistically inoculated with bacteria processed into a lactose based pellet, and one female elk group was subcutaneously inoculated with saline and served as a control group. The organisms used for ballistic inoculation were grown in a sterile fermentor previously used to grow smooth *Brucella* organisms. Serum samples were collected and cultured prior to vaccination and periodically until 18 weeks (bull group) or 27 weeks PI. In addition, multiple tissues were cultured from bull elk necropsied every two months after inoculation and one female elk necropsied after euthanasia due to a traumatic event. Seventeen elk isolates were originally recovered, but only ten isolates were found to be viable upon receipt in our lab. Of the ten viable isolates, six were recovered from the blood within the first approximately one and a half months and four were recovered from tissues no later than approximately seven months post-vaccination. When examined, all viable isolates belonged to the *Brucella* genus and lacked detectable levels of LPS associated O-chain. No differences could be found between the elk isolates and the original inoculums in bacteriological and biochemical tests indicating that RB51 is stable during passage through elk and after processing into lactose-based pellets.

Three selected elk-derived isolates were further examined by passing each through mice. Twenty-five BALB/c mice were divided into five groups of five mice each. These groups of mice were inoculated with the three isolates which were all from bull elk (thereby eliminating gender bias), were obtained at different times post-vaccination, and were recovered from more than one tissue source. The original culture of RB51 used to inoculate the elk and RB51 recovered from a biobullet were inoculated into one of each remaining mouse group. All mice received essentially equivalent doses (from 2.82×10^8 to 3.19×10^8 bacteria) and were bled prior to vaccination and at 2 and 4 weeks PI. Mice were euthanized and their spleens cultured at 4 weeks PI. Statistical analysis showed no significant difference between groups when considering the numbers of colony forming units cultured from the spleens of each mouse group. Since the persistence of bacteria in mice spleens can be an indication of a *Brucella's* virulence (106), all of the isolates or biobullet-processed RB51 appeared to have maintained the level of attenuation of the original RB51. When

examined bacteriologically and biochemically, all mice re-isolates were shown to belong to the *Brucella* genus and to lack detectable levels of O-chain.

The examination of both elk and mice isolates as described above indicated that RB51 is a very stable bacteria even after passage through two animal species. This is an extremely important characteristic since reversion to a smooth and more virulent form would eliminate one of RB51's greatest advantages over smooth vaccine strains, namely the lack of O-chain production. Since RB51 does not produce an O-chain portion on its LPS, vaccinated animals will not be detected in conventional diagnostic tests which are specific for anti-O-chain antibodies. However, a serological test which could easily and accurately identify RB51 vaccinated animals would be very helpful to animal producers and managers. Therefore, an ELISA to detect RB51 vaccinated elk was developed.

Both an indirect ELISA and a dotblot test were developed to identify elk with RB51-specific titers. After evaluating both tests and multiple forms of data analysis, an ELISA using elk serum diluted to 1:50 and a monoclonal antibody specific for bovine IgG_1 was found to be the most accurate, easily reproducible, and convenient method to evaluate elk serum samples. All ELISA optical density readings for each sample was converted into a percent positivity value for analysis. Percent positivity was defined as the ratio of the specific OD of the sample to the specific OD of a positive control. It is designed to represent where a sample lies along a straight continuum with zero binding of antibody being equivalent to 0% positivity and the antibody binding from the positive control being equivalent to 100% positivity. Therefore, the percent positivity of each sample represents the ratio of specific antibody in that sample to the amount of specific antibody in the positive control.

Through use of the saline control group data, a negative cutoff value was determined above which a sample would be considered to have a significantly elevated anti-RB51 antibody level. Using this rationale, all RB51 inoculated elk were shown to have an antibody response to vaccination. No saline inoculated elk had positive titers. Repeated measures analysis suggests that saline inoculated elk (non-vaccine exposed) could be tested at any time between four and twenty-seven weeks PI since all tested samples yielded negative test values. Both female and male subcutaneously inoculated were found to be serologically positive at four weeks PI (the first post-inoculation collection date). This sample date had the highest titer although an equally high or higher value may have been reached prior to this (samples not available). The ballistically inoculated group yielded the highest average titers at eight weeks PI, although they were also elevated at 4 weeks PI. Even though the percent positivity values for the ballistically inoculated group was consistently lower than for the two subcutaneously inoculated groups, the difference was not statistically significant. This suggests that processing RB51 into lactose based pellets and shooting them into elk (in this system) does not affect the antibody response.

Researchers associated with the University of Wyoming had reported serological reactions in the card agglutination test with elk serum after ballistic exposure suggesting that the RB51 organisms processed in the pellets had reverted to a smooth morphology. However, bacteriological examination of bacteria removed from pellets as well as all isolates recovered from ballistically inoculated elk failed to support this hypothesis. It is likely that any anti-O-chain antibodies detected by the card test would have been the result of LPS contamination of the equipment in which the RB51 organisms were grown.

Serum samples from each mouse group were evaluated in two separate ELISA studies. The objective of the first study was to determine if mice inoculated with elk-derived RB51 isolates or biobullet derived isolates would produce varying antibody levels against the original RB51. Statistical analysis revealed that the detectable anti-RB51 antibody responses at both two and four weeks PI did not significantly vary regardless of the isolate used. This indicates that the antigenic

profile of each isolate responsible for the induction of antibodies detectable in the ELISA is similar to that of the original culture of RB51. The objective of the second mouse ELISA study was to determine if passage through elk or processing into biobullets would cause an alteration in the antigenic profile of the RB51 bacteria. If so, different antibody levels could be detected when the same serum is tested against the original RB51 antigen and the appropriate isolate. Statistical analysis revealed no differences when testing samples against RB51 or the mouse's original inoculum further confirming the stability of the isolates and biobullet-processed bacteria.

Both of the mice ELISA studies as well as the elk ELISA study indicate that the developed ELISA protocol is useful for detecting RB51-vaccinated elk and mice and that the antigenic profile responsible for the induction of ELISA detectable antibodies of RB51 is highly stable. The development of the ELISA protocol is notable as no equivalent serological test have been described. Since this test is relatively easy to perform and can be standardized between laboratories, it could be commercially developed and used throughout the world. In elk, ELISA results suggest that the antigenic profile does not appear to be significantly altered when the bacteria is processed into biobullets. This finding is encouraging as the ballistic route of inoculation may be the only acceptable means of vaccinating elk on national feeding grounds without the complication of vaccinating non-target animals (which might occur with oral vaccinations).

The dotblot was developed concurrently with the indirect ELISA. Dotblots are most commonly interpreted subjectively by the researcher. This can lead to wide variations in sample results both over time and between laboratories. A method to interpret dotblot objectively was pursued with the use of an optical computer program (NIH Image). However, due to the lack of color uniformity within each dot and the extreme sensitivity of the program, standard deviations of the measured areas were often very large. Due to this and other complications, dotblot analysis of serum samples for RB51-specific antibodies was found to be unsatisfactory as compared to the ELISA.

To further study the humoral immune response of elk to RB51 vaccination, a random sample from each experimental group was examined through Western blot analysis. Both pre- and post-inoculation samples from each selected animal were tested against RB51 antigen and developed first using the monoclonal antibody to bovine IgG_1 used in the ELISA and then with polyclonal anti-bovine IgG antiserum. While multiple bands were noted in both pre- and post-vaccination samples, the only bands which appeared to be specific to RB51 vaccination were observed between 6 and 17 kDa after development with the anti-IgG_1 monoclonal. Bands observed above this range and after development with polyclonal IgG antisera are likely the result of cross-reactivity of RB51 antigens with antigens from other bacteria to which the elk are exposed to in nature. Therefore, the development of antibodies to antigens between 6 and 17 kDa should be further studied as a means of identifying RB51 vaccinated elk.

In vaccinated mice, a similar banding pattern among the five groups indicate that regardless of the source of the RB51 inoculum (original culture, biobullet, or elk-derived), the mice produce anti-RB51 antibodies specific for the same antigens of RB51. Small variations in the number of bands observed with each group may be a result of slightly higher or lower antibody levels developed by individuals within a group.

When the humoral immune response to RB51 in mice is further examined in Western blot, differing isotype and sub-isotype antibodies appear to predominate. IgG_{2a}, IgG_{2b}, and IgG_3 isotype antibodies are produced against many different antigens in RB51 and likely compose the predominate antibody response. Mice IgG_1 isotype antibodies appear to be produced against only one RB51 protein while IgM antibodies do not appear to participate as detected in this test. The lack of IgM antibodies may however be the result of problems with the IgM antisera. Overall,

RB51 vaccination appears to stimulate an antibody response in mice composed of multiple isotypes and sub-isotypes.

Antigen segments which produce the highest antibody response in infected animals could be used in a commercial ELISA for easy and uniform testing of animals. However, it should be noted that the antibody detected in the ELISA most likely has little or no long-term protective effects as immunity induced by RB51 vaccination to *B. abortus* is regulated mostly by the cell-mediated immune response.

In summary, this study developed a highly reliable ELISA which can detect RB51 vaccinated elk using a monoclonal antibody to bovine IgG_1 and acetone killed whole RB51 bacteria. It is possible that the antibodies detected in the ELISA are directed against antigens of a molecular weight between 6 and 17 kDa.

This study also indicates that RB51 is a highly stable strain which does not revert to smooth morphology or initiates synthesis of LPS-O-chain, maintains it biochemical characteristics, does not undergo detectable antigenic variations, and remains attenuated after successive passages in elk and mice. Its ability to be processed into biobullets without detectable antigenic alterations makes it extremely valuable for use in the vaccination of wildlife. Overall, the results of this research indicates that RB51 is a potentially valuable vaccine for the prevention of Brucellosis in elk. Further studies will have to be performed to determine its protective value in this species.

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APPENDIX - Recipes of Solutions Used

Christensen's urease test media
1g peptone
5g sodium chloride
2g monopotassium phosphate
0.012g phenol red
1g dextrose
20 g agar
1 L distilled water
adjust pH to 6.8

Crystal Violet Stock Solution
2 g crystal violet
0.8 g Ammonium Oxalate
20 ml Absolute Ethanol
80 ml Distilled water

Crystal Violet Working Solution
Dilute stock 1:40 v/v in distilled water.

Bicarbonate phosphate solution (coating buffer)
1.59 g Na_2CO_3
2.93 g $NaHCO_3$
bring to a final volume of 1 liter
titrate to 9.6 pH

Phosphate buffered saline - Tween 20 (PBST-20)
8 g NaCl
0.2 g KH_2PO_4
0.9 g Na_2HPO_4
0.2 g KCl
0.5 ml Polyoxyethylenesorbitan Monolaurate (Tween 20)
titrate to 7.4 pH

0.18 M H_2SO_4 to stop ELISA substrate development:
4.5 ml 8N H_2SO_4
95.5 ml distilled water

Tris buffered saline - 10X concentration (TBS)
48.4 g Tris
584.4 g NaCl
bring to a final volume of 2 liters
titrate to 7.5 pH

Tris buffered saline - Tween 20 - 10X concentration (TBST)
48.4 g Tris
584.4 g NaCl
6 ml Tween 20

bring to a final volume of 2 liters
titrate to 7.5 pH

SOLUTIONS USED IN SDS-PAGE:

Resolving Gel Buffer
3M Tris-HCl
36.6 g Tris base
48 ml 1M HCl
bring to a final volume of 100 ml with distilled water
titrate to pH 8.8
filter
store at 4˚ C for no longer than one month

Resolving Gel (12.5% Acrylamide)
for two 1.5mm thick mini-gel
4.68 ml 40% acrylamide stock
1.9 ml resolving gel buffer
0.15 ml 10% SDS
7.57 ml HPLC-grade water
0.75 ml 1.5% ammonium persulfate
7.5 ul TEMED

Reservoir Buffer Stock (10X concentration)
0.25M Tris, 1.92M glycine, 1% SDS
30.3 g Tris base
144.0 g glycine
10.0 g SDS
dissolve in distilled water and bring to a final volume of 1 liter
dilute 1:10 to use
store at 4˚ C

Stacking Gel Buffer
0.5M Tris-HCl
6 g Tris base
20 ml distilled water
titrate to pH 6.8 with 6M HCl
bring to a final volume of 100 ml with distilled water
filter
store at 4˚C for no longer than one month

Stacking Gel
0.94 ml 40% acrylamide stock
2.5 ml stacking gel buffer
0.1 ml 10% SDS
5.96 ml HPLC-grade water
0.5 ml 1.5% ammonium persulfate
10 ul TEMED

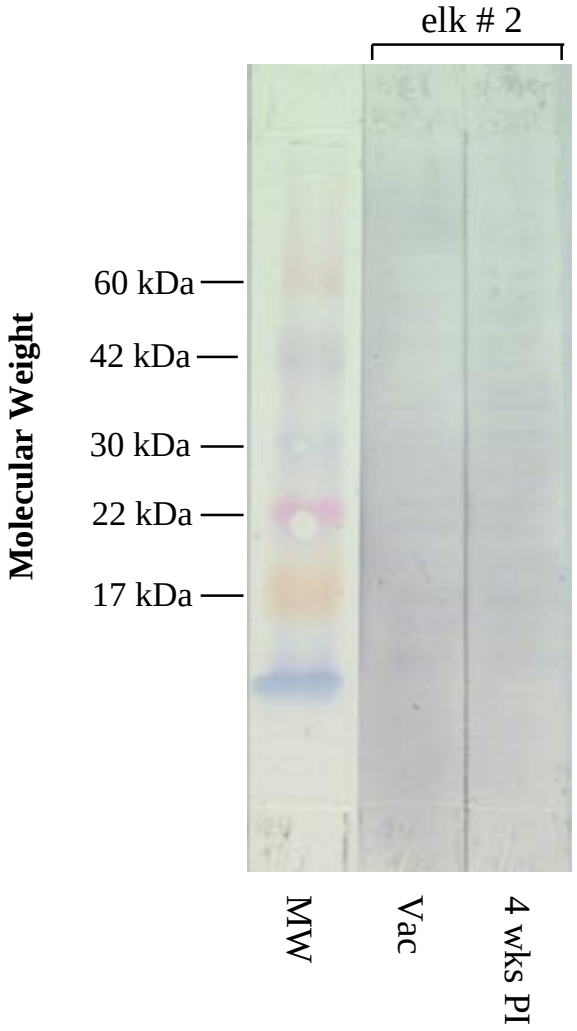

Figure 2.11. Western blot of saline elk #2 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA)

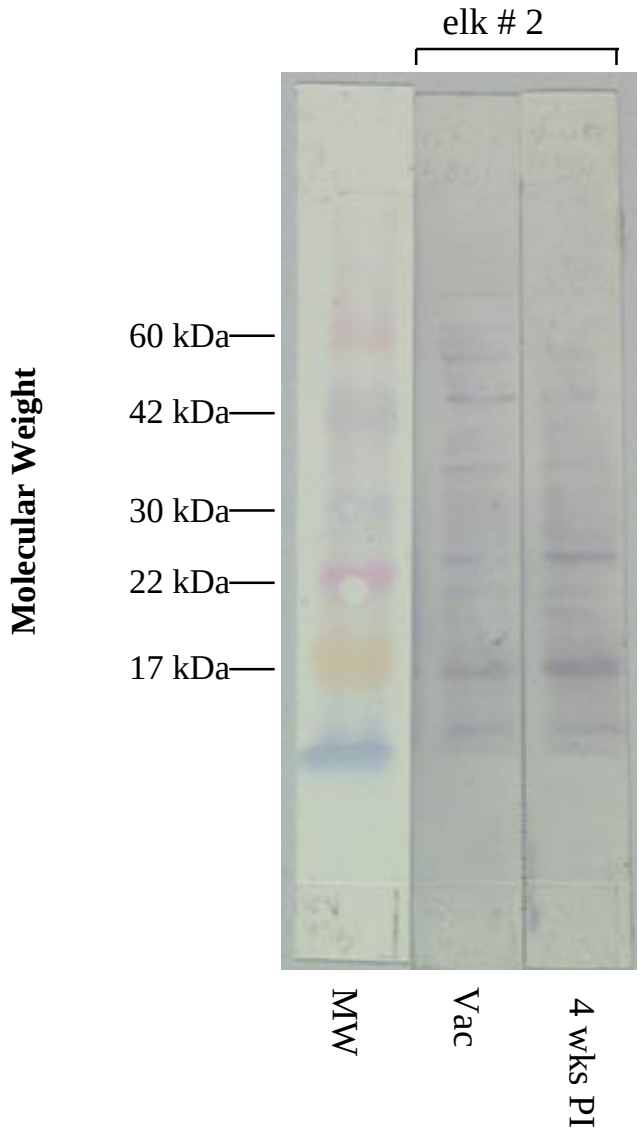

Figure 2.12. Western blot of saline elk #2 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

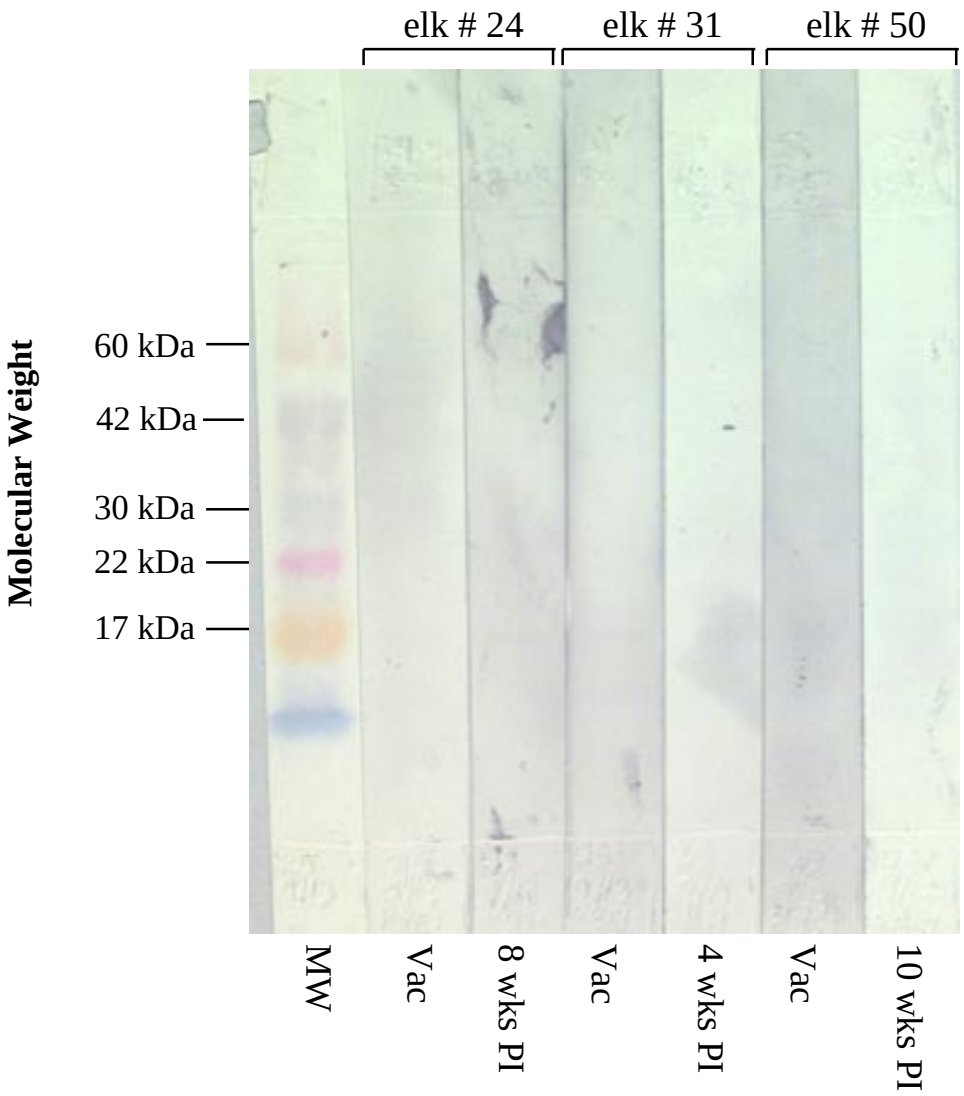

Figure 2.13. Western blot of saline elk #24, 31, and 50 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

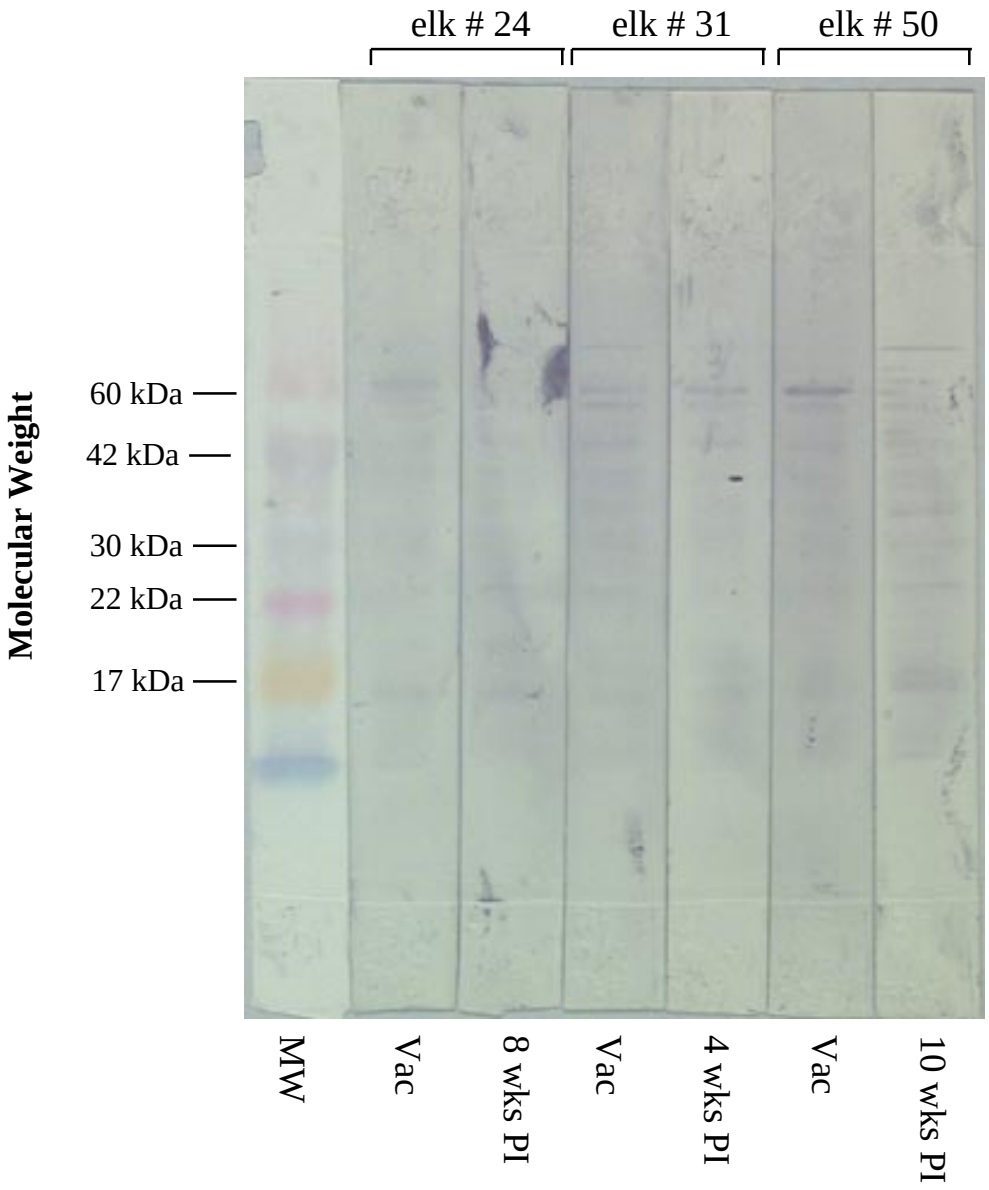

Figure 2.14. Western blot of saline elk #24, 31, and 50 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel-West Chester, PA).

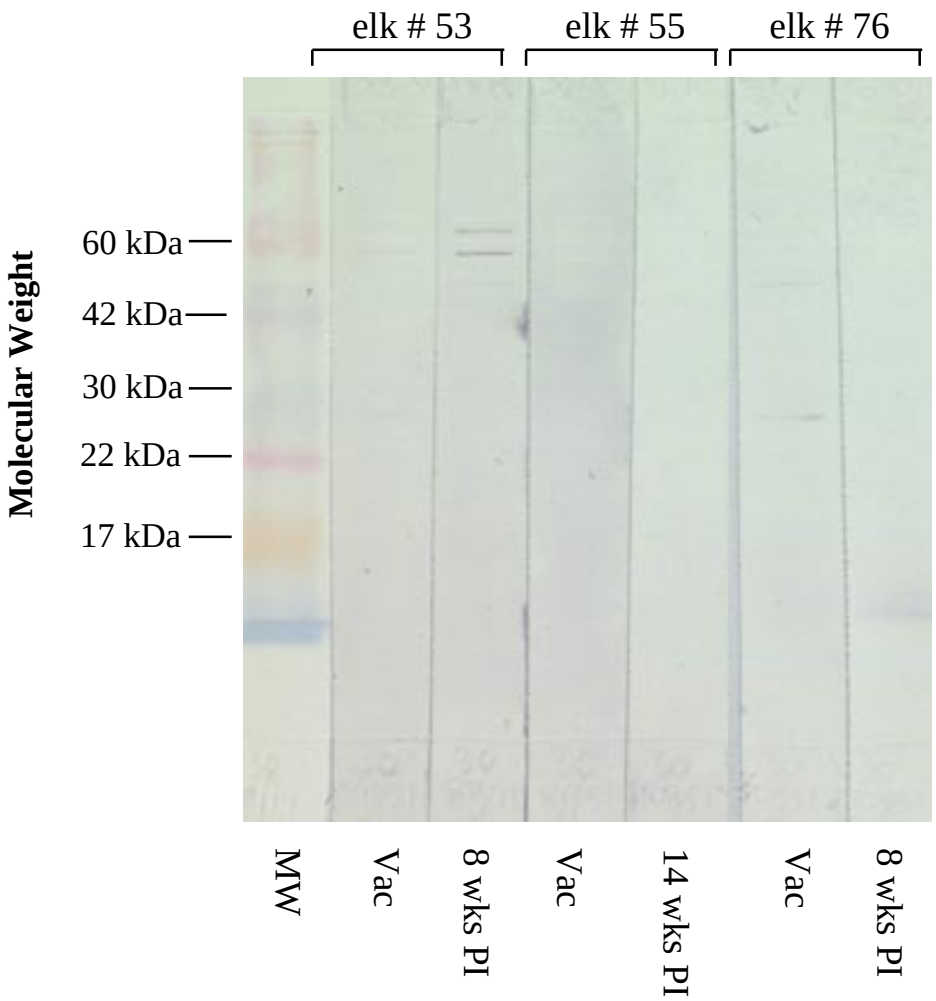

Figure 2.15. Western blot of saline elk #53 and 55 and biobullet elk #76 against whole cell RB51 antigen developed with monclonal anti-bovine IgG1 (VMRD - Pullman, WA).

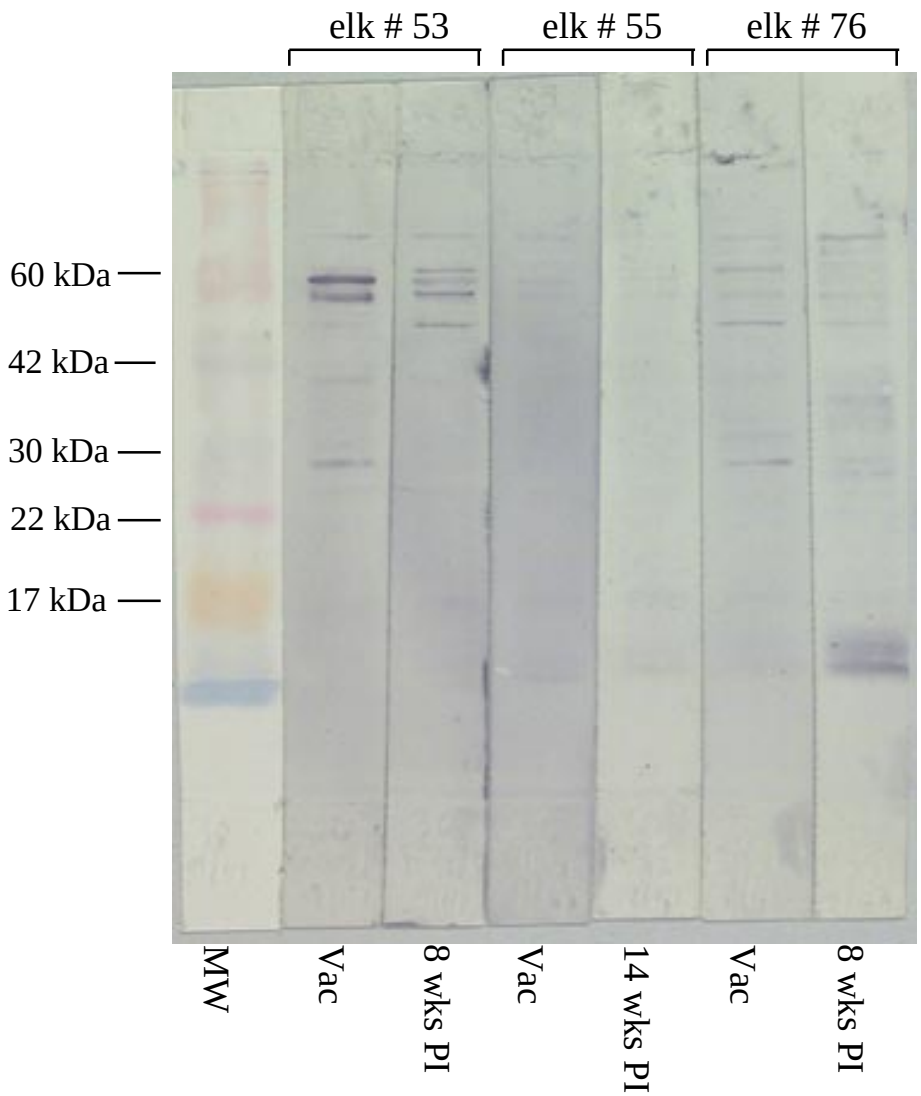

Figure 2.16. Western blot of saline elk #53 and 55 and biobullet elk #76 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel-West Chester, PA).

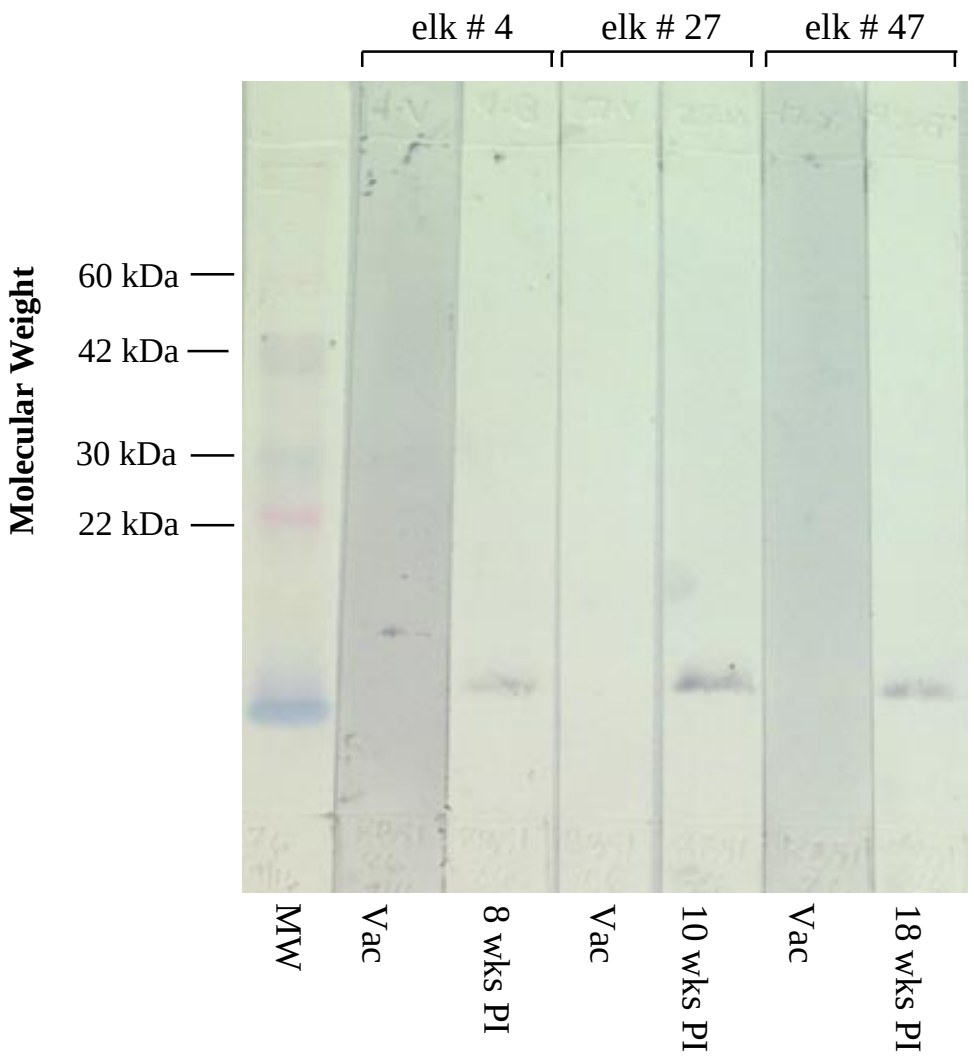

Figure 2.17. Western blot of biobullet elk #4, 27, 47 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

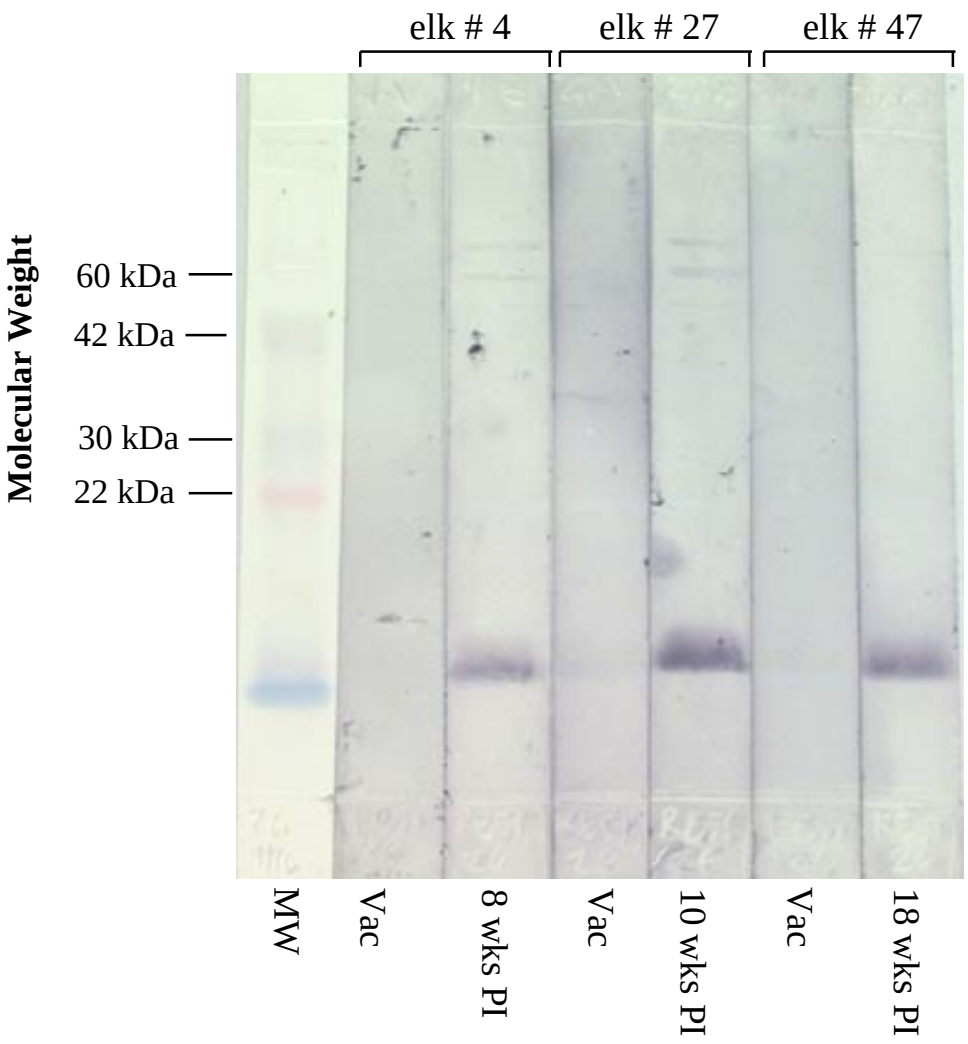

Figure 2.18. Western blot of biobullet elk #4, 27, and 47 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

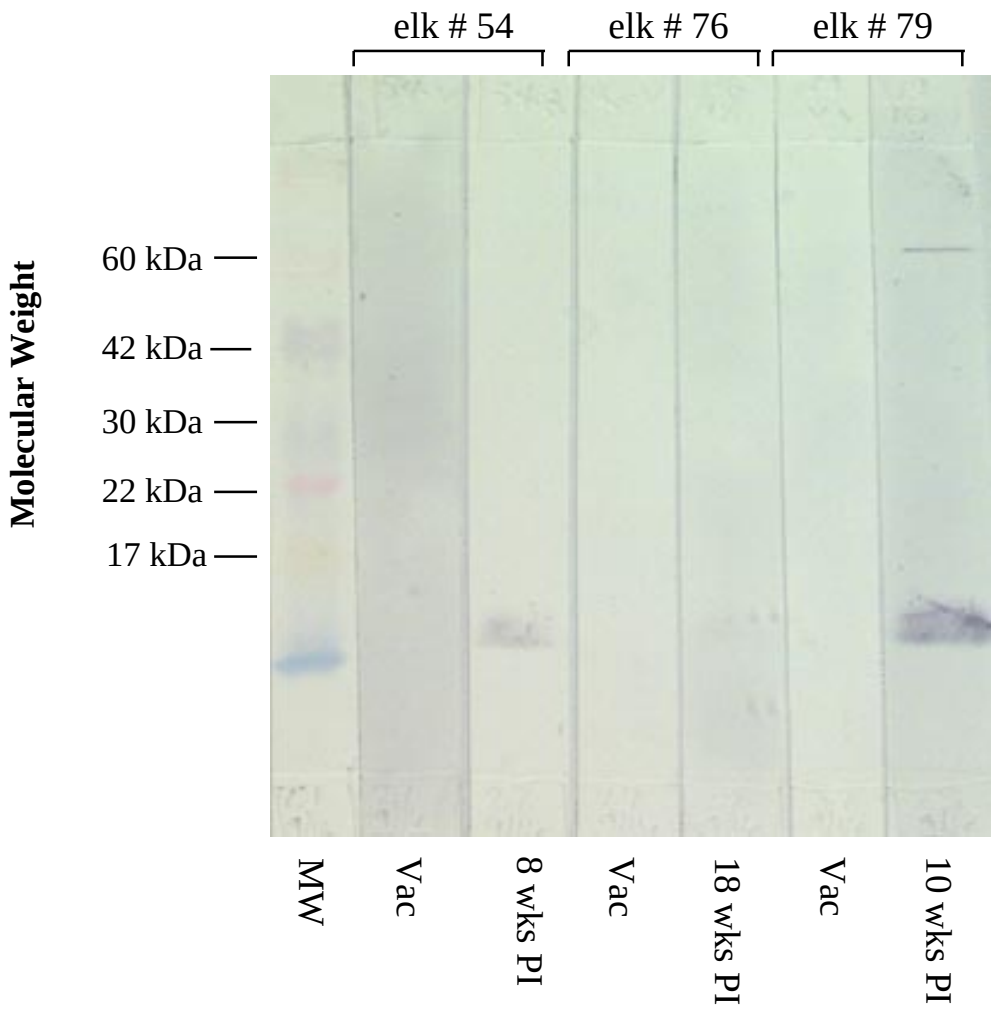

Figure 2.19. Western blot of biobullet elk #54, 76, and 79 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

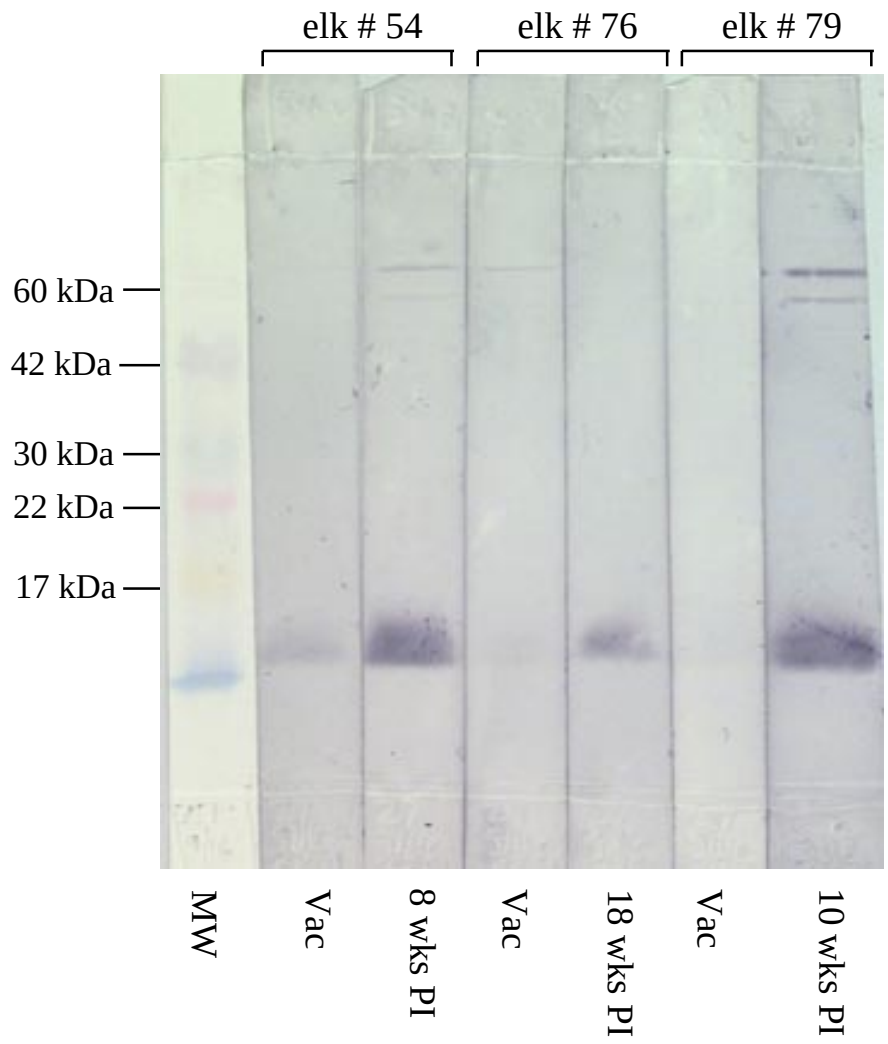

Figure 2.20. Western blot of biobullet elk #54, 76, and 79 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

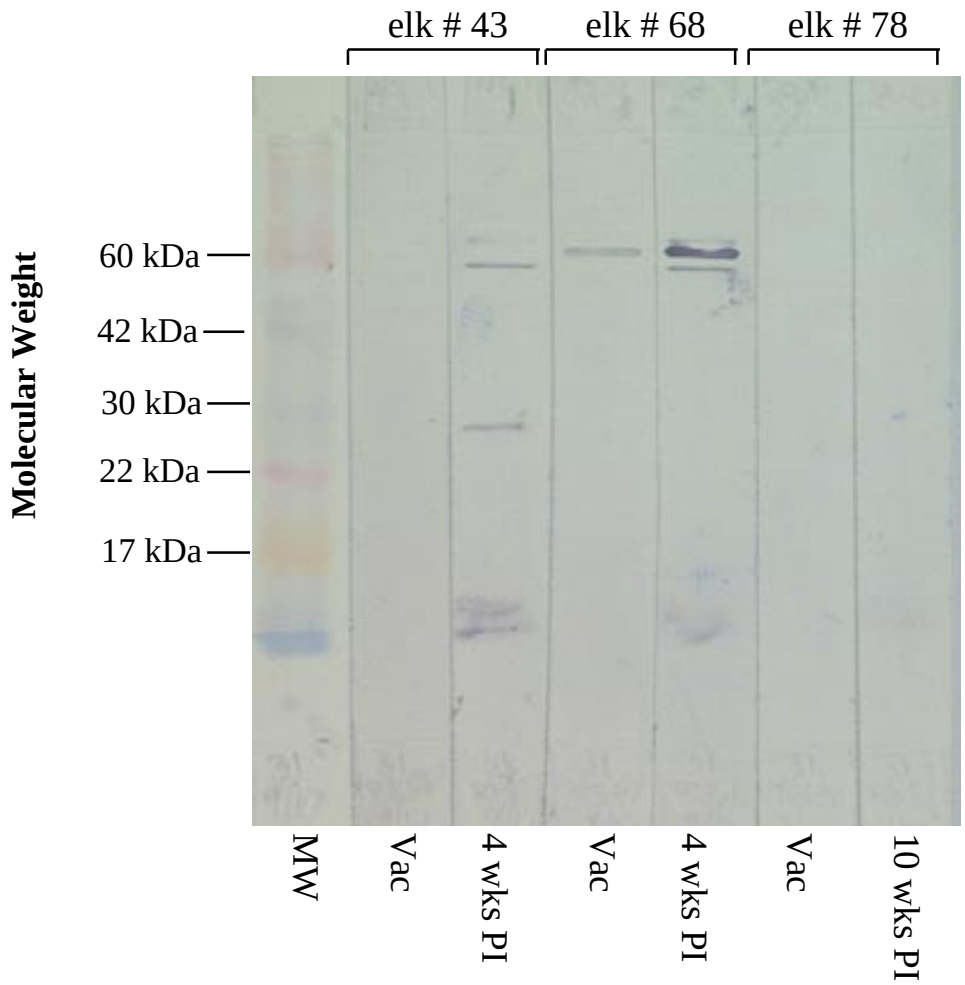

Figure 2.21. Western blot of female subcutaneously inoculated elk #43, 68, and 78 reacted against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (Pullman, WA).

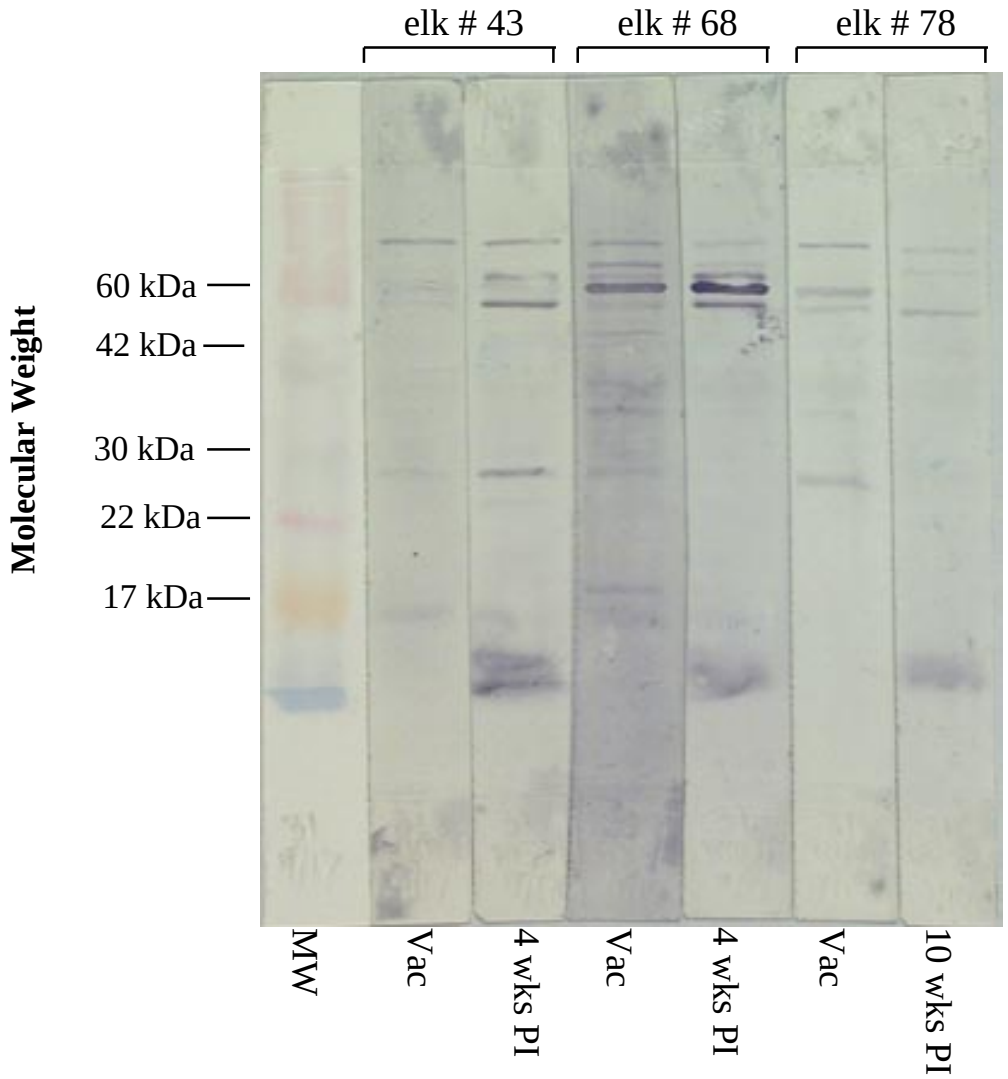

Figure 2.22. Western blot of female subcutaneously inoculated elk #43, 68, and 78 reacted against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel-West Chester, PA).

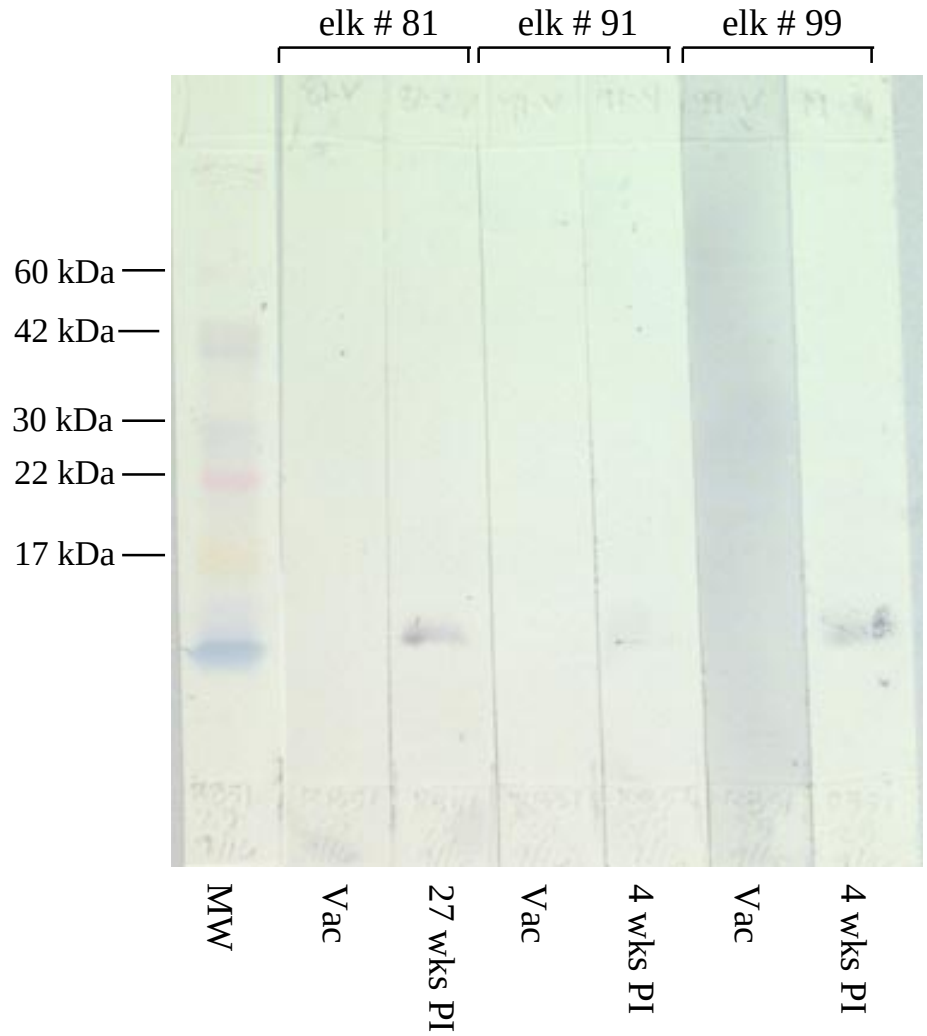

Figure 2.23. Western blot of female subcutaneously inoculated elk #81, 91, and 99 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

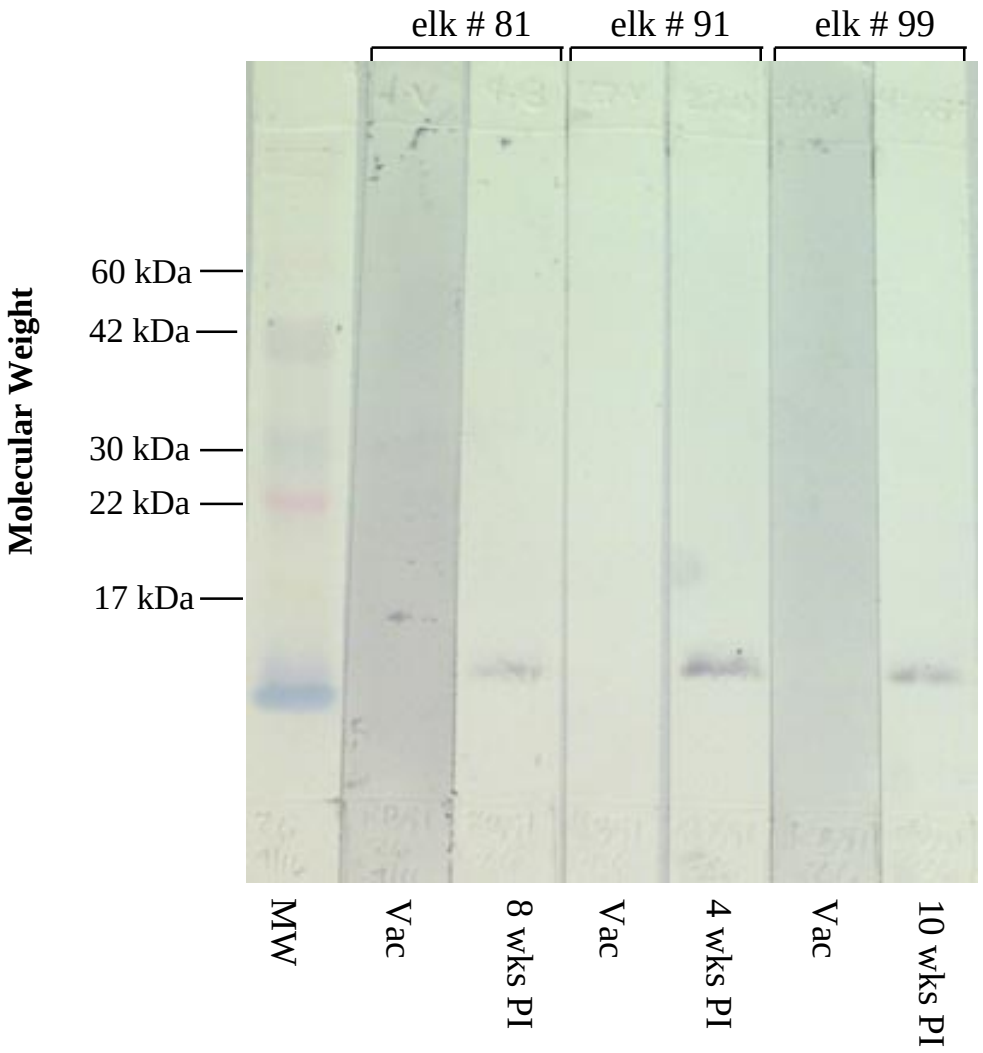

Figure 2.24. Western blot of female subcutaneously inoculated elk #81, 91, 99 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

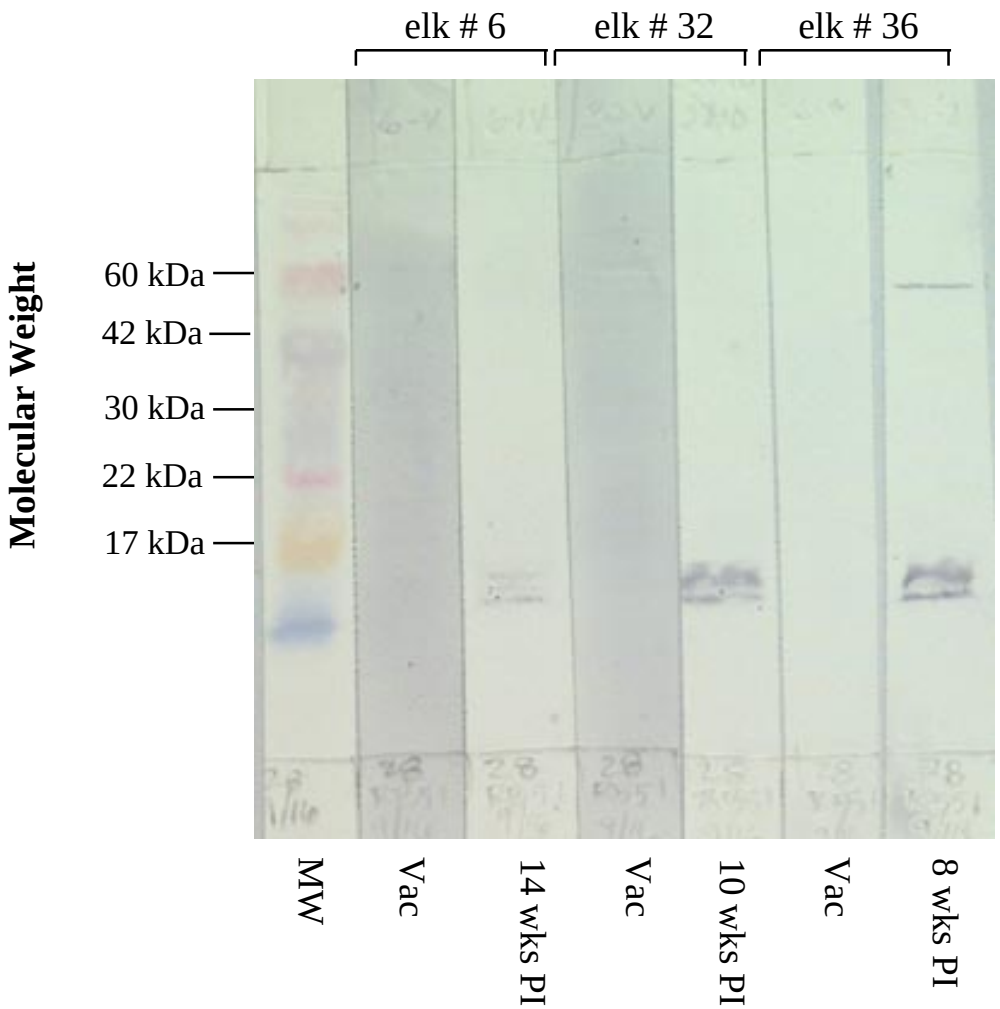

Figure 2.25. Western blot of bull subcutaneously inoculated elk #6, 32, and 36 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

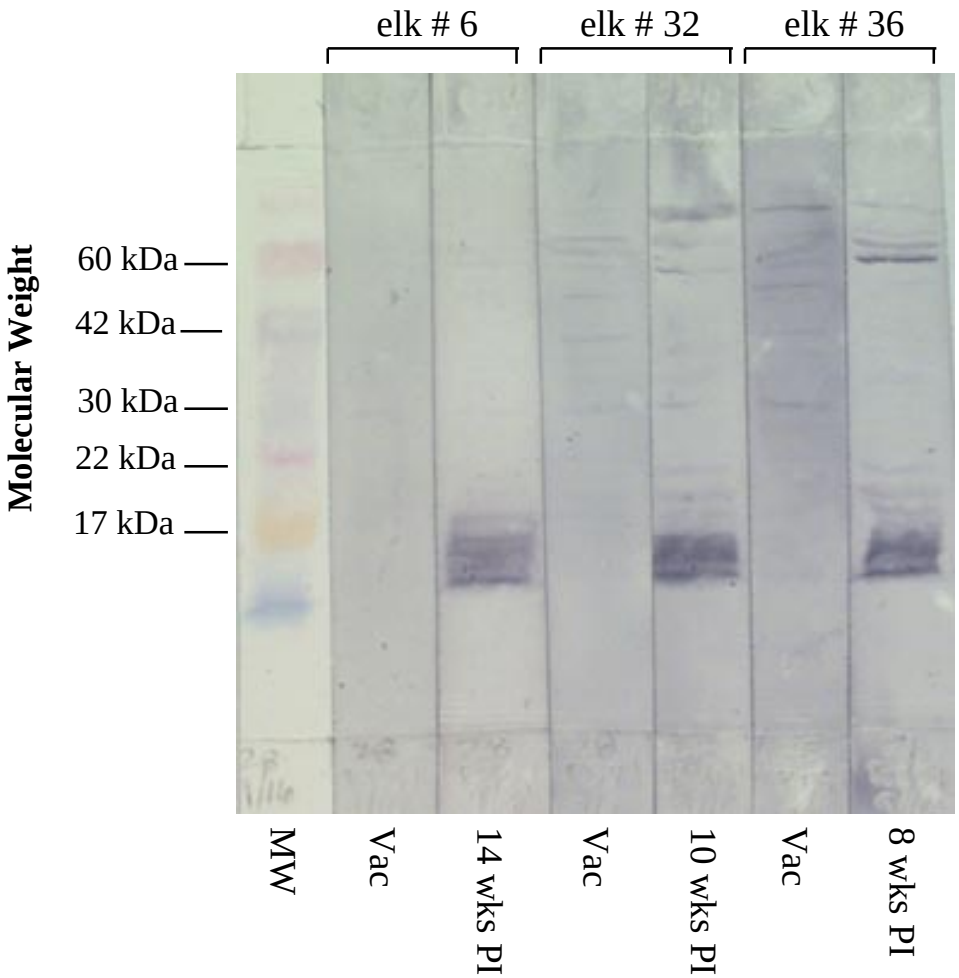

Figure 2.26. Western blot of bull subcutaneously inoculated elk #6, 32, and 36 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

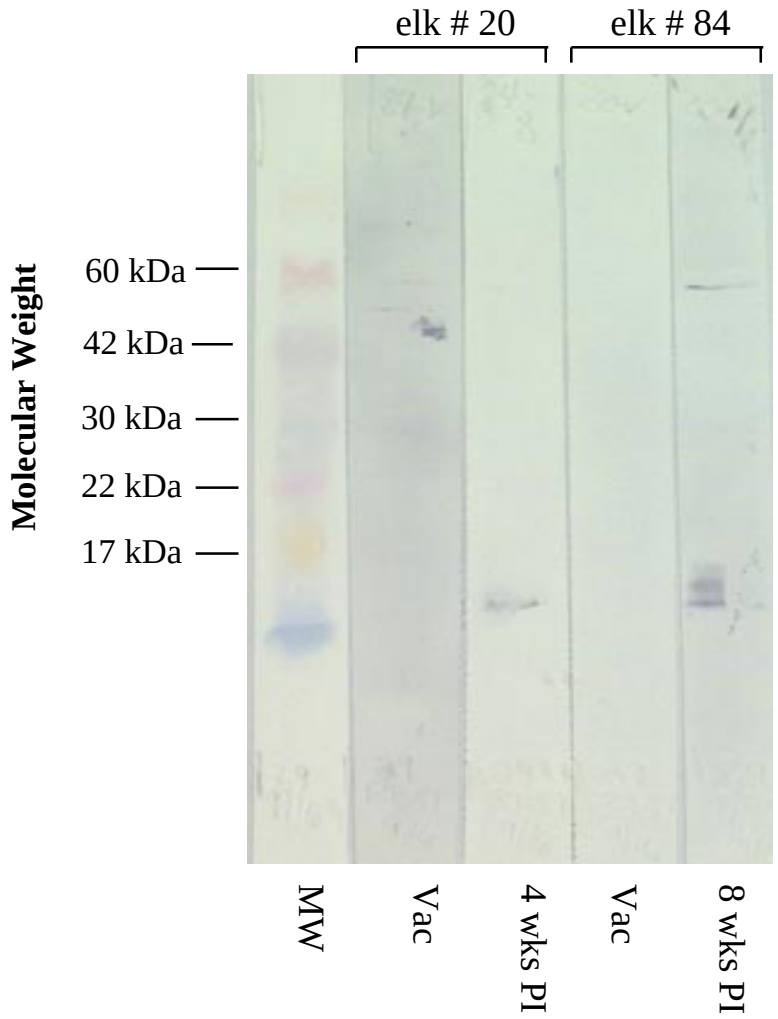

Figure 2.27. Western blot of bull subcutaneously inoculated elk #20 and 84 against whole cell RB51 antigen developed with monoclonal anti-bovine IgG1 (VMRD - Pullman, WA).

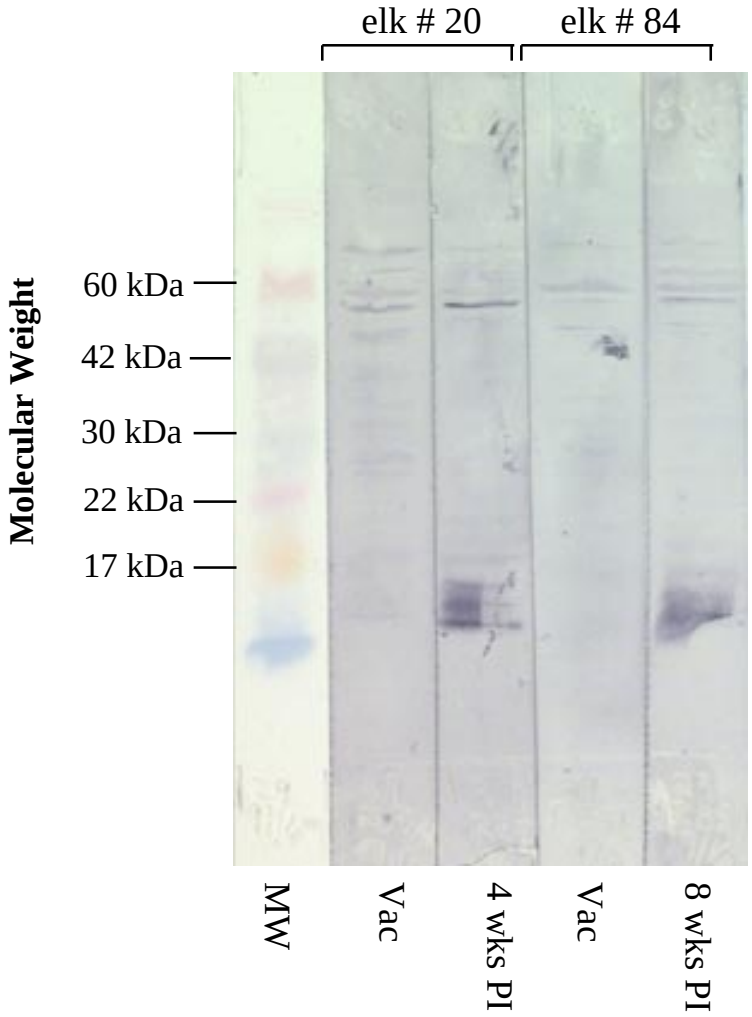

Figure 2.28. Western blot of subcutaneously inoculated bull elk #20 and 84 against whole cell RB51 antigen developed with polyclonal anti-bovine IgG (Cappel - West Chester, PA).

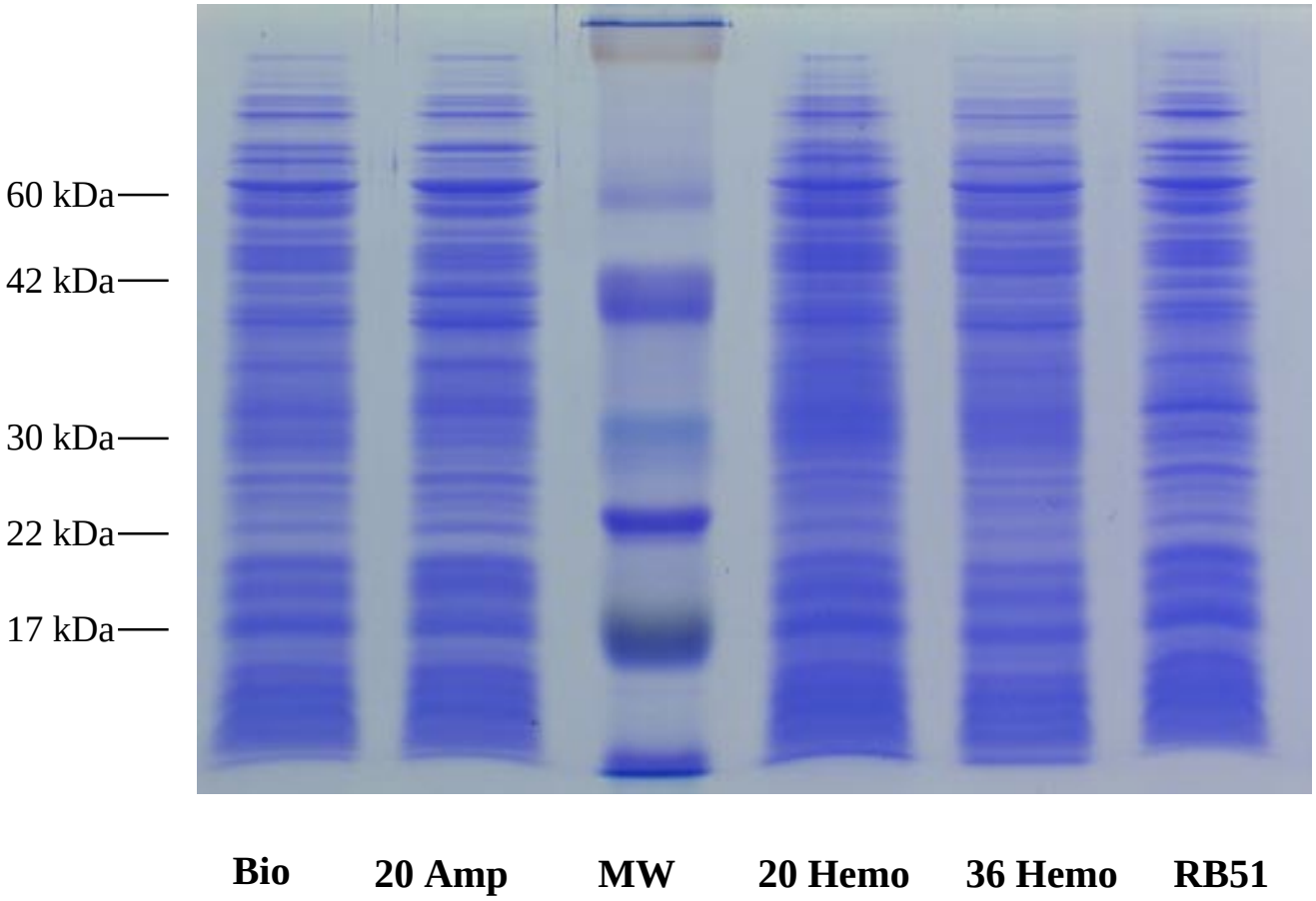

Figure 3.1. SDS-PAGE of selected elk isolates, RB51, Biobullet and molecular weight standard.

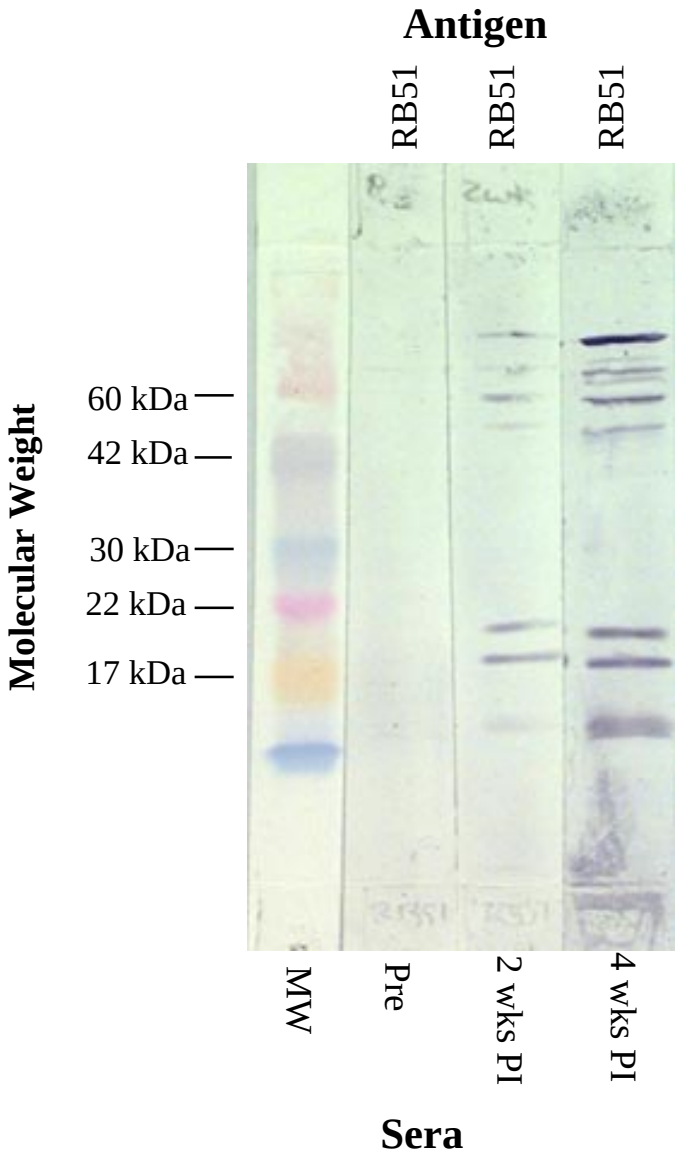

Figure 3.2. Western blot with pooled sera (pre-inoculation, 2 weeks post inoculation, and 4 weeks inoculation) from the RB51 mouse group reacted against whole cell RB51.

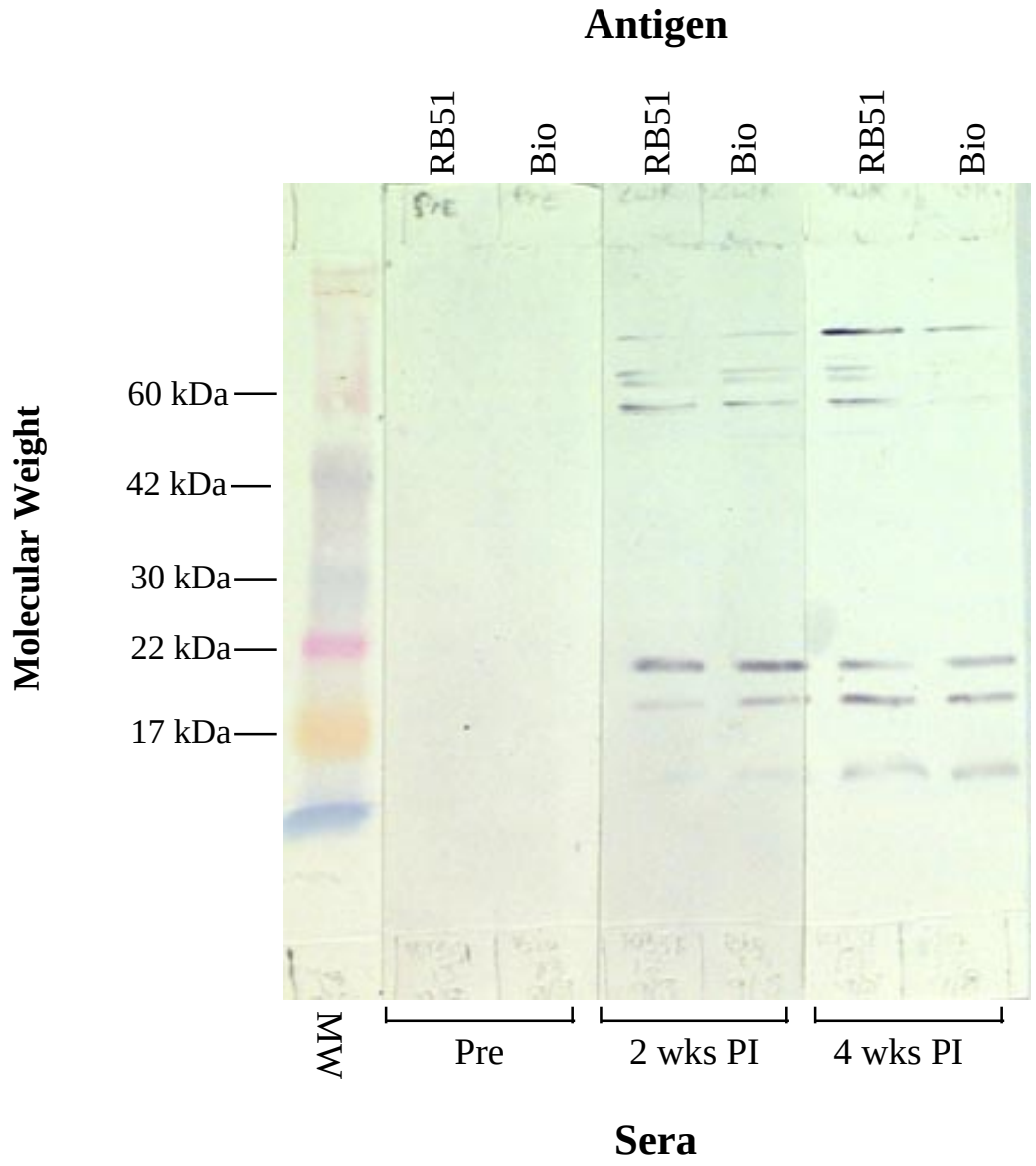

Figure 3.3. Western blot with pooled sera (pre-inoculation, 2 weeks post inoculation, and 4 weeks inoculation) from the Biobullet mouse group reacted against whole cell RB51 and Biobullet isolate antigens.

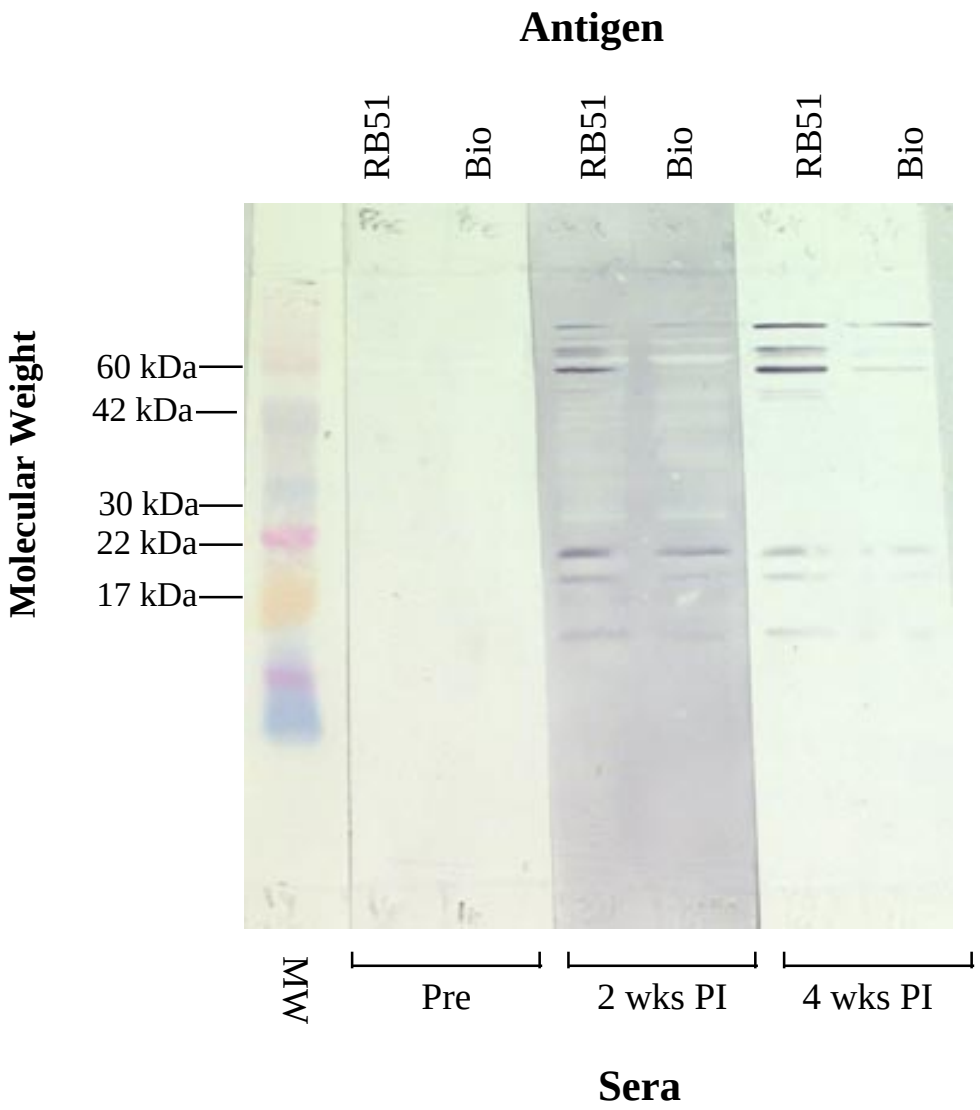

Figure 3.4. Western blot with pooled sera (pre-inoculation, 2 weeks post inoculation, and 4 weeks inoculation) from the elk #20 ampulla mouse group reacted against whole cell RB51 and elk #20 ampulla isolate antigens.

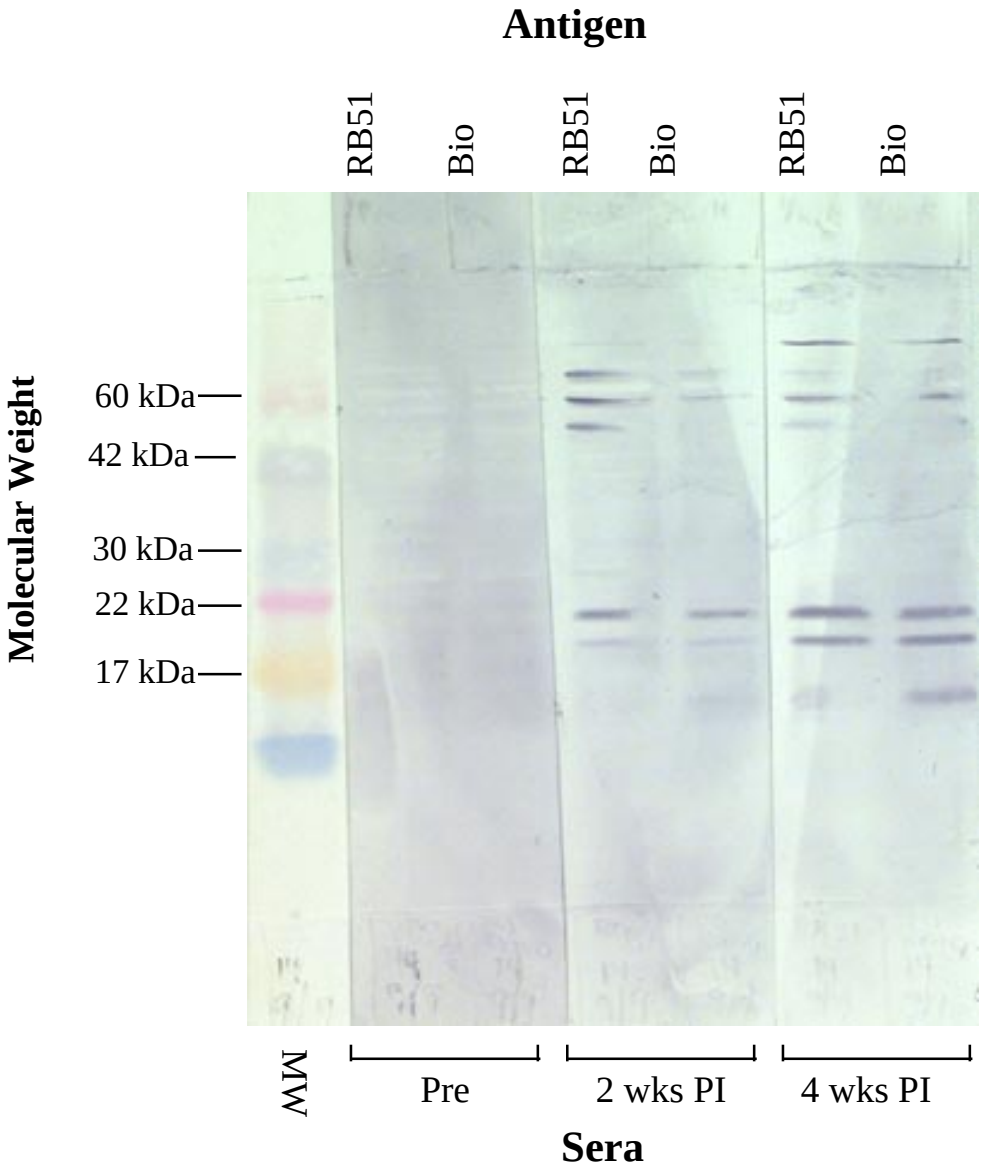

Figure 3.5. Western blot with pooled sera (pre-inoculation, 2 weeks post inoculation, and 4 weeks inoculation) from the elk #20 hemoculture mouse group reacted against whole cell RB51 and elk #20 hemoculture isolate antigens.

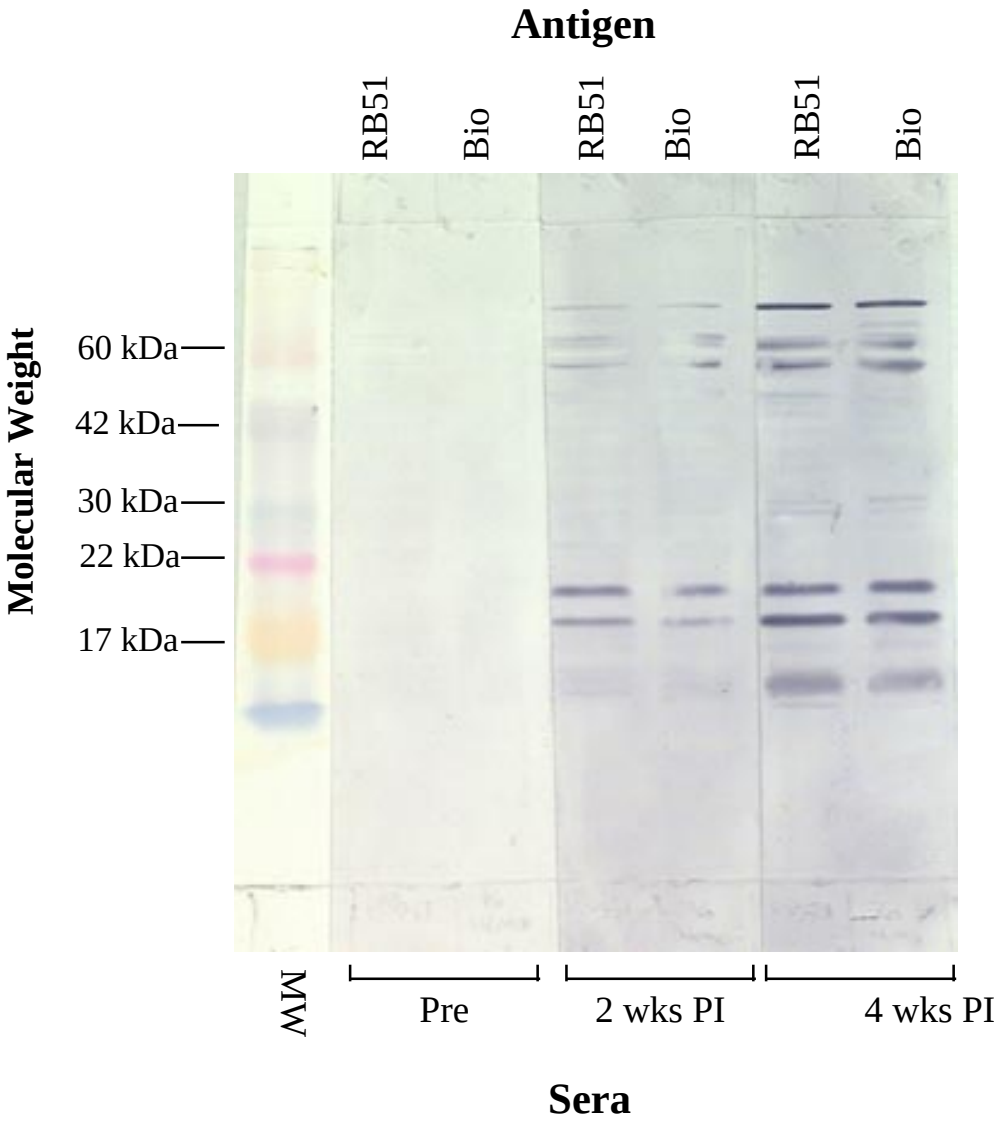

Figure 3.6. Western blot with pooled sera (pre-inoculation, 2 weeks post inoculation, and 4 weeks inoculation) from the elk #36 hemoculture mouse group reacted against whole cell RB51 and elk #36 hemoculture isolate antigens.

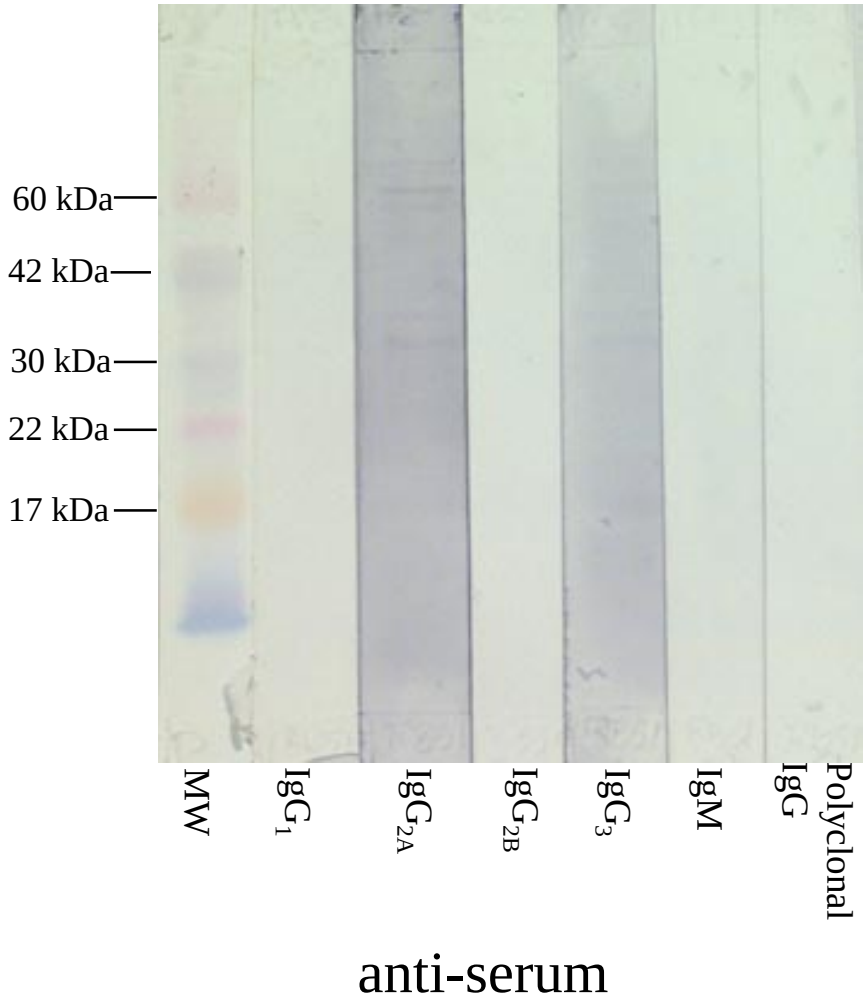

Figure 3.7 Western blot of RB51 vaccinated mouse serum obtained prior to vaccination reacted with RB51 antigen and developed with different (sub)isotype anti-sera.

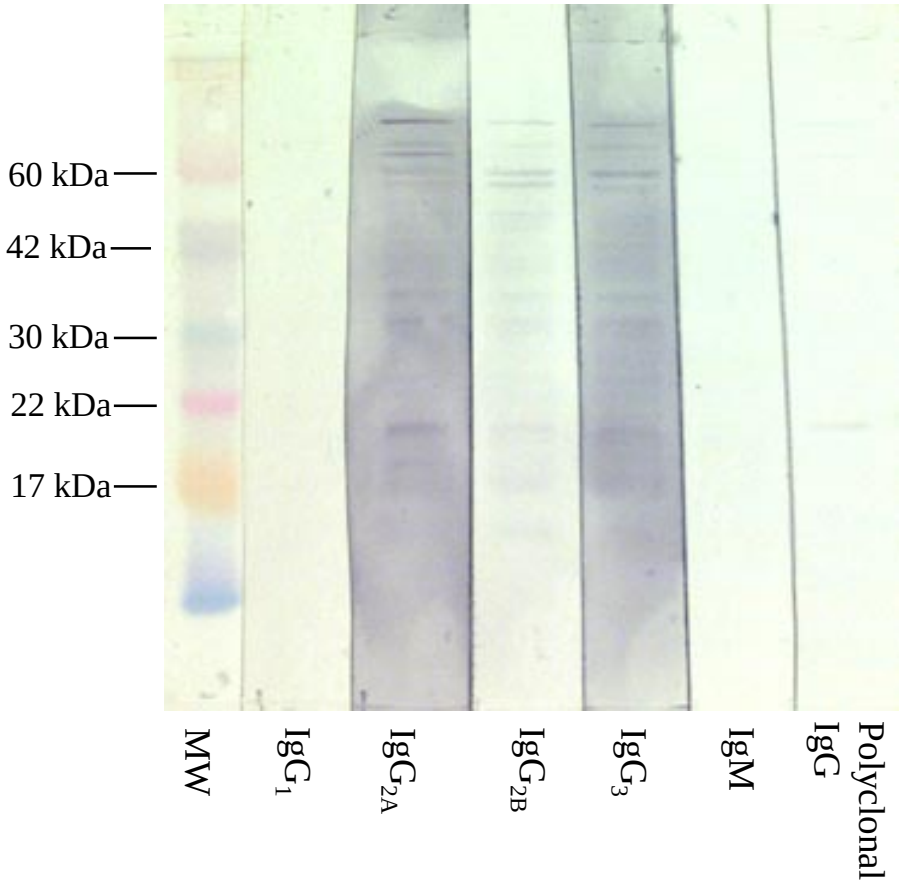

Figure 3.8 Western blot of RB51 vaccinated mouse serum obtained 2 wks PI reacted with RB51 antigen and developed with different (sub)isotype anti-sera.

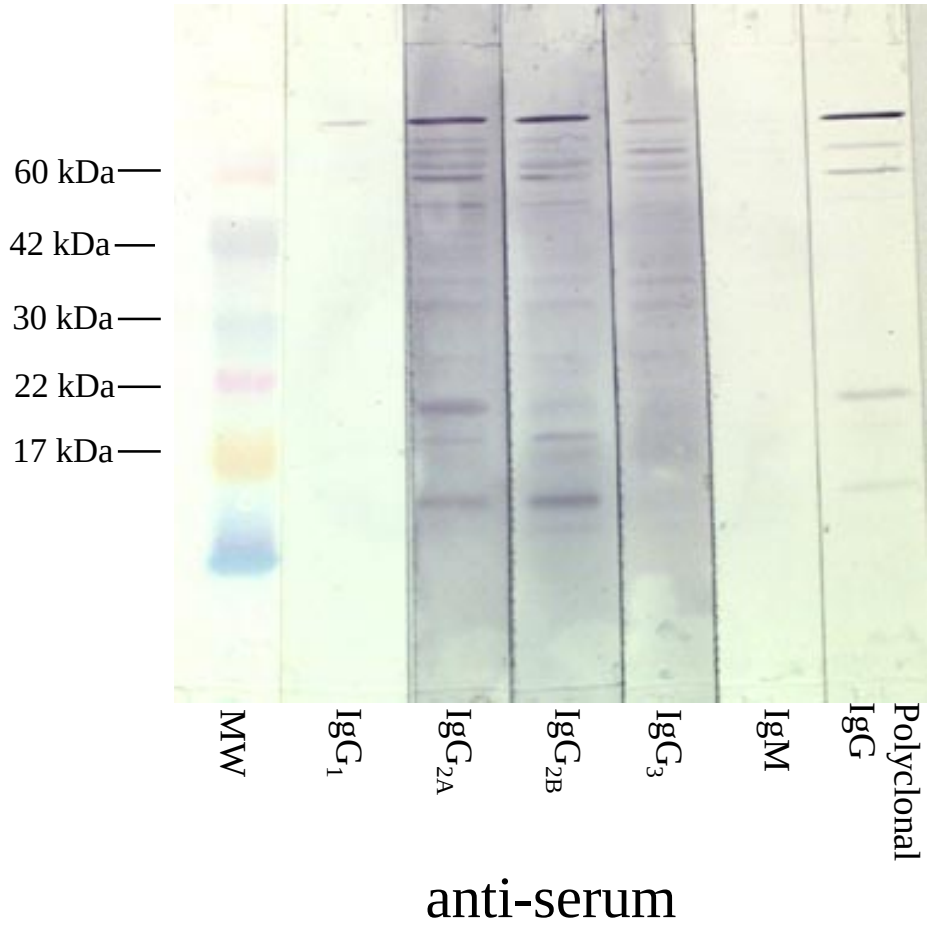

Figure 3.9 Western blot of RB51 vaccinated mouse serum obtained 4 wks PI reacted with RB51 antigen and developed with different (sub)isotype anti-sera.

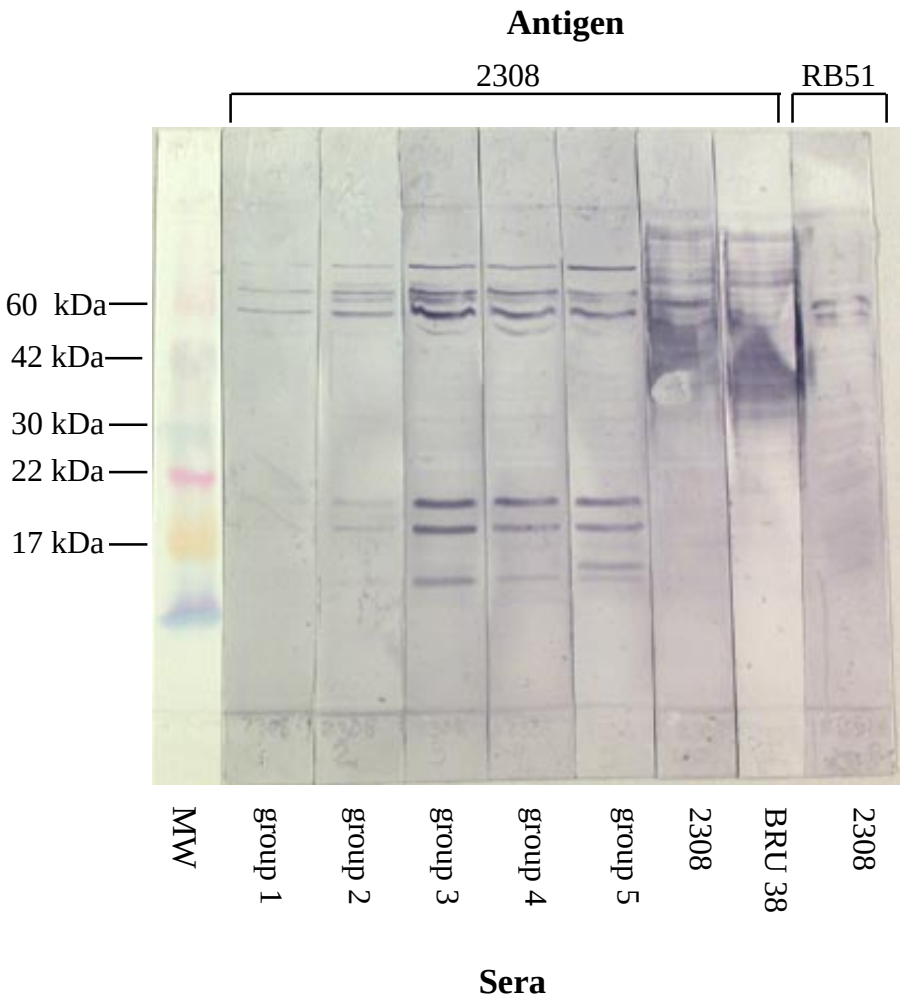

Figure 3.10. Pooled sera obtained (two weeks post inoculation) from each mouse experimental group reacted with whole cell *B. abortus* strain 2308 and developed with horse radish peroxidase conjugated polyclonal IgG anti-mouse. The right-most three strips served as controls. Note that no mouse groups appeared to react with the LPS region of the 2308 antigen. 2308 serum was obtained from a strain 2308 immunized mouse.

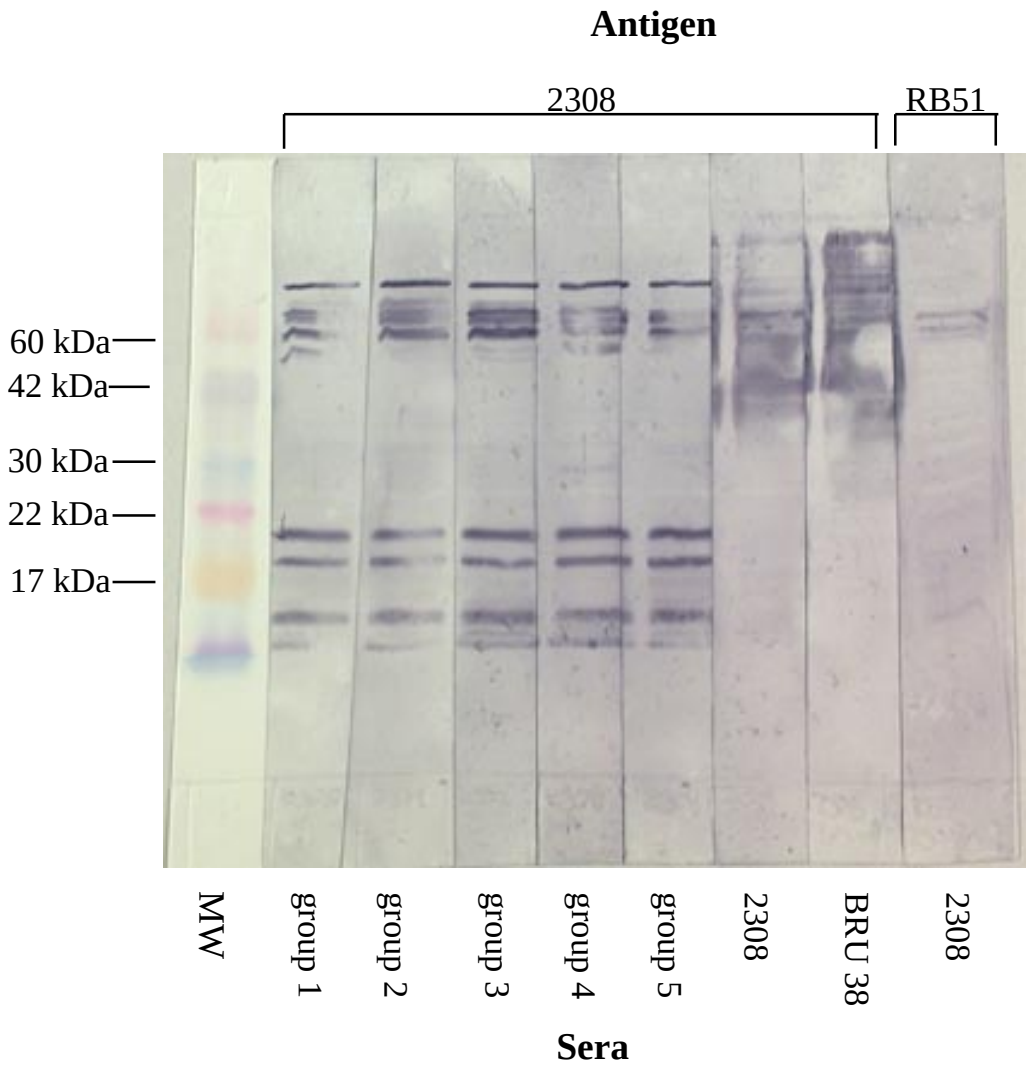

Figure 3.11. Pooled sera obtained (at four weeks post inoculation) from each mouse experimental group reacted with whole cell *B. abortus* strain 2308 and developed with horse radish peroxidase conjugated polyclonal IgG anti-mouse. The right-most three strips served as controls. Note that no mouse groups appeared to react with the LPS region of the 2308 antigen. 2308 serum was obtained from a strain 2308 immunized mouse.

Lesley A. Colby DVM, MS

Education:

Doctor of Veterinary Medicine, May 1996
Master of Veterinary Science, *The Humoral Immune Response of Elk (Cervus elaphanus nelsoni) and Mice against Vaccination with Brucella abortus Strain RB51,* February, 1997
Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM)
Blacksburg, VA 24061

Professional License: State of Virginia Veterinary License
State of Montana Veterinary License - pending

B.S. Animal Science, May 1992
Virginia Polytechnic Institute and State University (VPI & SU)
Blacksburg, VA 24061

Select Sires Artificial Insemination Short Course, January 1995

Experience:

Research Assistant, VMRCVM, Blacksburg, VA
Dates: summers of 1993–1995 and May 1996 – February 1997
- Developed an indirect ELISA to detect RB51 vaccinated elk.
- Conducted serological testing through antigen preparation, indirect ELISA development, immunoblots (SDS-PAGE, Western blot, and Dotblot), gel electrophoresis, count electrodiffusion, and agglutination tests (tube and slide).
- Identified bacterial isolates from animals through bacterial culturing and traditional biochemical testing.
- Worked with infectious materials (*Brucella spp*.) in a Biosecurity Level 3 lab.

Smith-Kilborne Foreign Animal Disease Training Program, Foreign Animal Disease Diagnostic Laboratory, Plum Island, NY
USDA / APHIS 1/94
- Attended lectures on foreign animal disease diagnosis, epidemiology, control, and eradication.
- Observed and necropsied food animals and birds infected with foreign animal diseases.

Agricultural Laboratory Technician B, VPI & SU Blacksburg, VA
Dates: 5/91–8/91
- Independently tracked radio-tagged Bald Eagles using radio-telemetry while flying, boating, or hiking in the Chesapeake Bay area.
- Fitted juvenile Bald Eagles with USFWS leg bands and radio transmitters.
- Responsible for project house, vehicle, boat and two temporary volunteers.

Agricultural Laboratory Technician A, VPI & SU Blacksburg, VA
Dates: 8/90–5/91
- Examined food samples for Piping Plover Study.
- Helped feed and care for 23 Dunlin shorebirds.
- Organized research materials.

Undergraduate Technician, VPI & SU Blacksburg, VA
Dates: 5/90–8/90
- Researched affects of phenols and tannins on grouse.
- Dissected ducks and quail to determine fat deposition.
- Organized research data on raccoon diets.

Undergraduate Technician, VPI & SU Blacksburg, VA
Dates: 1/90–8/90
- Assisted in surgery (hysterectomies) of swine.
- Assisted with radioimmunoassays.

Senior Year Veterinary Preceptorships:

Companion Animal Medicine, Companion Animal Surgery, Food Animal and Equine Medicine and Surgery, Laboratory Services, Radiology VMRCVM.

Epidemiology Summer Program offered by the New England Epidemiology Institute Boston, Massachusetts June 5 – 23, 1995.

courses taken: Introduction to Epidemiology, Theory and Practice of Epidemiology II, Clinical Research, and Epidemiology in Developing Countries.

Viral and Rickettsial Zoonoses Branch, Centers for Disease Control
Atlanta, Georgia October 23 – December 3, 1995.
- co-authored: "Rabies in rodents and lagomorphs in the United States, 1985–1994: Temporal and spatial associations between woodchucks (*Marmota monax*), raccoons (*Procyon lotor*) and other animals." Childs, Colby, Krebs, Strine, Feller, Noah, Drenzek, Smith, and Rupprecht. Accepted in Journal of Wildlife Diseases 9/96.
- assisted Epidemic Intelligence Service officers in gathering and interpreting epidemiological data.

Production Management Medicine, Iowa State University
Aimes, Iowa January 25 – February 9, 1996.
- studied techniques in swine medicine and production

Veterinary Medical Center, mixed animal practice (90% large/10% companion animals)
Williamsburg, Iowa February 12 – March 8, 1996.
- accompanied food animal veterinarians on sick animal calls and herd health visits.

University of Idaho Sheep Experiment Station
Dubois, Idaho March 18 – April 14, 1996.
- assisted station veterinarian with preventative and therapeutic treatment of sheep flock during lambing season.

Department of Veterinary Science, University of Wyoming
Laramie and Jackson Hole, Wyoming February 26 – March 17, 1996.
- participated in domestic and wild animal necropsies and histological examinations.
- assisted in trapping, darting, handling, bleeding, and transporting elk from feeding grounds to be used in Brucellosis research.

Awards:

John Vaught Senior Scholarship, 1995

Virginia Veterinary Medical Association Auxiliary Award, 1994

Simmon Eyre Wildlife Scholarship, 1993

John L. Pratt Senior Scholar, 1991–92

American Society of Animal Science, Scholarship Award 1991 and 1992

Professional Memberships:

Virginia Veterinary Medical Association 1996-present

Student Chapter of the American Veterinary Medical Association
- President 1994, President-Elect 1993

Student American Veterinary Medical Association Delegate, 1/94–1/95

Wildlife Disease Association, 1995–present

American Association of Wildlife Veterinarians, 1995–present

Food Animal Practitioners Club, VMRCVM, 1992–95

Wildlife and Exotic Animal Club, VMRCVM, 1992–95

VPI Cave Club, President 3/90–3/91; Treasurer 3/89–3/90