DIAGNOSIS, EPIDEMIOLOGY AND IMMUNOLOGIC CONSEQUENCES OF COPPER DEFICIENCY IN CALVES

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by
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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

DIAGNOSIS, EPIDEMIOLOGY AND IMMUNOLOGIC CONSEQUENCES OF COPPER DEFICIENCY IN CALVES

Presented by Ronald Kenneth Tessman

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance.

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DEDICATION

As we travel through life, we start as simple vulnerable entities that gather strength and confidence as we gain experience. We all hope some day to become great at something. But no matter how much experience we have or confident we become, there will always be times where we revert to that sweet, vulnerable entity we used to be. It is at those times that we fall back and depend on those that have influenced us the most, those that have helped us come this far. It is not often that we share credit for our victories with these people.

In my life for sure there have been many people whose influence have carried me this far. This victory, this dissertation is dedicated to four of those individuals; my Grandmother, Ruby Eich, my Grandfather, Jerry Eich, my Dad, James Daniels, and my Best Friend, Maury Reichelt.

When I think of my grandma, my mind always takes me to a lonely, scared boy of three standing by a chain link fence crying for his grandmother, worried that he will never see her again and not understanding why he is in this place with all these orphans. She saved me. She was my greatest protector, toughest critic and disciplinarian, and my best example of unconditional love. Grandma, although it has been many years since your passing, I still remember your lessons daily. I wash my hands, cook my meat, say yes ma'am and yes sir, hold the door open for whoever's behind me and love with all my heart.

My grandpa had an easy going, pleasant outlook on life. He was always positive and generous. He would give you the last dollar in his pocket and think nothing of it. He had such an excellent work ethic that he figured he could just work harder and make more money the next day. He was never scared to confront a new challenge. He would just look at the situation and tackle it head on. He was my best example of hard work, generosity, and way of being. Grandpa, I hope that I have made you proud. I will always remember your sayings. They mean more now to me then they did then. “If you’re early to work you’re on time, and if you’re on time you’re late.” “Ronnie, at some point you’re going to have to take responsibility for your own education. Some people learn that lesson early and some people never learn that lesson.”

Dad, when I was young and scared, I never dreamed that I would have a Dad like you. When I met you, you surpassed all my expectations. You are more than I hoped for and represent everything I want to be. You are strong, both physically and mentally. You walk unafraid of what life may bring. You are generous to a fault. Money does not hold the same place of importance to you as it does to so many in this life. For you, success is measured by happiness not by the size of your bank account. You have the biggest heart of any person I have ever met. You love your family and friends with a passion that not many understand. I will try to live by your statement, “The only person that scares me is me.” I love you and hope to be more like you.

Maury, I have known you longer than anyone I can remember. You have always been there, like a solid rock. We have seen so much together that I can't imagine what life would have been without you. There is not an aspect of my life you haven't touched. I know if it weren't for you I probably would have never attended undergraduate let alone veterinary and graduate school. I look forward to the years to come and the experiences we will share. You truly are my brother, and I love you.

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Dr. Krause, your pragmatic and honest approach to statistics is enlightening. I have always loved numbers and your guidance has helped to harness that love into something useful. Your ability to convey the meaning of statistics and stimulate interest is one I intend to develop.

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CHAPTER 1

COPPER DEFICIENCY IN CATTLE

OVERVIEW

Copper is an essential component of several enzymes, required to maintain host homeostasis. Included are superoxide dismutase, cytochrome oxidase, lysol oxidase, ascorbic acid oxidase and ceruloplasmin (Larson et al., 1995; Smart et al., 1992). Additionally, adequate copper nutrition is required for iron metabolism and the prevention of cellular oxidative damage. Copper deficiency has been linked to variety of clinical disease manifestations. These include pale hair coat, decreased fleece quality, anemia, spontaneous fractures, poor capillary integrity, myocardial degeneration, hypomyelinization of the spinal cord, impaired reproductive performance, decreased resistance to infectious disease, diarrhea and generalized ill-thrift (Gay et al., 1988; Gooneratne et al., 1989; Smart et al., 1992). Clearly, none of these clinical presentations are pathognomonic. These clinical signs may be confused with other diseases. Consequently, many producers and veterinary practitioners fail to recognize copper deficiency. Cattle with copper deficiency have generalized, but non-specific ill-thrift and impaired productivity. This decreased productivity is the most significant economic loss associated with copper deficiency (Larson et al., 1995; Suttle, 1986 a).

COPPER DEFICIENCY DISEASE MANIFESTATIONS

Pale hair coat is a direct result of a decrease in available copper, affecting the activity of the enzyme, tyrosinase (Movaghar, 1989). This reduction in activity results in

both morphologic and functional changes of melanocytes. This functional change is directly responsible for both the change in hair color and hair shaft fragility or wool break that is a classic result of copper deficiency described in sheep (Patterson et al., 1974).

Diarrhea due to copper deficiency results from structural and functional changes in mitochondria secondary to a relative decrease in cytochrome oxidase (Fell et al., 1975). These changes result in morphologic changes of the intestinal mucosa characterized by mucosal and villous atrophy accompanied by crypt elongation and goblet cell hyperplasia. The overall effect is one that makes the intestine less efficient in absorption and leads to diarrhea as well as emaciation (Mills, 1976).

The role of copper deficiency in orthopedic diseases is well described. For competent cartilage formation and subsequent bone mineralization lysyl oxidase is required (Kosonen et al., 1997). Copper is an essential component of this enzyme. In the absence of adequate lysyl oxidase activity fragility of long bones occurs. Deficient animals are far more likely to suffer from long bone fractures than are copper replete animals (Jonas et al., 1993). In addition irregular thickening of the physis joint deformation is often observed. The formation of these structures in fast growing animals is further complicated by a relative decrease in copper dependent ATPase which serves a vital role in energy metabolism in these structures (Rucker et al, 1998). In addition copper deficiency has been linked to osteochondrosis lesions in young, fast growing horses (Bridges et al., 1984). In these instances overzealous supplementation with mineral high in zinc concentration resulting in copper deficiency has been identified as the inciting cause (Campbell-Beggs et al., 1994).

In many areas of the world that depend on small ruminants for subsistence neurological disease manifestations of copper deficiency are common (Faye et al., 1991;

Ivan et al., 1990). Two separate disease syndromes that represent a continuum of pathologic consequences have been described. Sway back and enzootic ataxia, result from what is described as hypomyelination of the spinal cord. Clinical signs vary from newborns unable to rise to an ataxia that progresses in severity as the animal grows (Kavanagh et al., 1972; Lewis et al., 1974). These both result from an oligodendrocyte dysfunction. Decreases of in utero cytochrome oxidase and superoxide dismutase allow for excessive oxidative damage in the growing fetus and effect oligodendrocyte maturation. This ultimately results in a decreased in myelin production (Sweasey and Patterson, 1979; Matsushima and Morell, 2001). Animals may appear normal at birth, but as growth occurs, loose the ability to make a competent synaptic response, resulting in ataxia followed by death (Lewis et al., 1974).

Copper deficiency dependent anemia is best described in human patients and rat experimental models (Gyorffy and Chan, 1992; Johnson and Hove, 1986; Hayton et al., 1995). There are a number of factors that can bring about anemia secondary to copper deficiency. This anemia is often termed a secondary iron deficiency anemia. Decreases in ceruloplasmin, lead to a decrease in iron oxidation and the ability of iron to move from the liver to bone marrow where it can be incorporated into hemoglobin and red blood cells. In addition, the second step of heme synthesis is catalyzed by ALA dehydrase a copper dependent enzyme. In copper deficiency this enzyme is decreased resulting in decreased heme synthesis and abnormal red blood cell formation (Brewer, 1987). More recent experiments in rats and mice support observations of increased erythrocyte fragility and decreased survivability. There are changes in the erythrocyte cytoskeleton that result in increased cell viscosity due to lipid loading (Johnson and Kramer, 1987; Jain and Williams, 1988).

COPPER STORAGE AND MOBILIZATION

The liver functions as the copper storage organ. Copper is liberated from hepatic storage to maintain blood copper concentrations and support essential host functions (Bingley and Dufty, 1972; Gooneratne et al., 1989; Linder and Hazegh-Azam, 1996). Consequently, blood copper concentration overestimates body copper stores in deficient states and underestimates body copper stores in intoxication states. In copper intoxication, intake exceeds demand. Liver storage far exceeds mobilization, resulting in net hepatic accumulation of copper (Gooneratne et al., 1989; Linder and Hazegh-Azam, 1996). However, blood copper concentrations are maintained within relatively narrow range of normal concentrations. When hepatic stores reach a critical threshold, acute hepatic necrosis results, releasing hepatic copper stores, causing transient high blood copper concentrations (Gooneratne et al., 1989; Linder and Hazegh-Azam, 1996). As a direct consequence, blood copper determinations will not permit diagnosis of copper intoxication unless sampling is coincidental with acute hepatic disease.

If ruminants are placed on copper deficient diets, hepatic stores will be mobilized to maintain blood copper concentrations and support vital host functions. Blood copper concentrations are maintained within a relatively normal range while hepatic copper concentrations slowly decline (Gooneratne et al., 1989; Suttle, 1986 b). These patterns of storage, mobilization and utilization directly impact our interpretation of blood copper concentrations. A high blood copper concentration is suggestive of copper intoxication with coincidental release of hepatic copper. A normal blood copper concentration is consistent with either high hepatic copper stores without coincidental hepatic copper release, normal hepatic copper stores, or low hepatic copper stores which are being

mobilized to maintain blood copper concentration. Low blood copper concentrations suggest that liver copper stores have been exhausted and an animal is in a deficient state (Suttle, 1986 b).

RISK FACTORS FOR COPPER DEFICIENCY

Risk factors for copper deficiency in cattle include decreased soil and diet copper concentrations, increased dietary or soil concentrations of either molybdenum, sulfates or iron, diets based on pasture rather than stored forages or grains, alkaline soils, and animal age (Campbell et al., 1974; Larson et al., 1995). Calves on pasture are generally limited to diets consisting of dam's milk and grass pastures. Both are poor copper sources (Naveh et al., 1981; Whitelaw, 1985). Young, rapidly growing cattle have much higher copper requirements (Gay et al., 1988; Smart et al., 1992; Whitelaw, 1985).

A recent survey of North American forages conducted for the USDA by the NAHMS group classified 14.2% of samples as deficient and 49.7% as marginal in copper content. High molybdenum and iron concentrations also were observed frequently in these analyses. Both will impair copper utilization (Corah and Dargatz, 1996). We have recently completed a statewide survey of blood copper levels of beef calves in Missouri. Our results indicate that copper deficiency is common in Missouri. About 20% of the calves sampled were copper deficient.

DIAGNOSIS OF COPPER DEFICIENCY

The diagnosis of copper deficiency is typically based on either tissue or blood copper concentrations. Plasma copper concentrations of less than 57 ug/dl are suggestive of

marginal copper deficiency and affected calves often demonstrate sub-optimal productivity (Larson et al., 1995; Whitelaw, 1985). Plasma copper concentrations less than 19 ug/dl are usually associated with more obvious signs of clinical deficiency (Smart et al., 1992; Whitelaw, 1985). Forage or soil concentrations of copper, sulfates, and molybdenum may be helpful adjuncts in the recognition of copper deficiency. Normal soils have copper concentrations of 18-22 ppm and deficient soils generally have copper concentrations less than 2 ppm (Whitelaw, 1985). Soil molybdenum concentrations greater than 10 ppm will often cause secondary copper deficiency (Gooneratne et al., 1989; Whitelaw, 1985). The complexity of interactions between copper and its inhibitors (iron, sulfates, molybdenum, soil pH) renders accurate prediction of copper deficiency based on soil or diet composition problematic if not impossible (Suttle, 1986 b; Smart et al., 1992). Many instances of potential copper deficiency are eventually diagnosed on the basis of clinical trials; Herds in which clinical disease incidence decreases or productivity improves following copper supplementation are presumptively diagnosed as having been copper deficient (Smart et al., 1992; Suttle, 1991; Suttle, 1986 b; Wikse et al., 1992).

Copper salts may be added to feed, mineral, and salt sources. Unfortunately, optimal supplementation requires consistent daily intake and salt and mineral supplements have highly variable daily intakes (Larson et al., 1995; Smart et al., 1992). This variability in intake makes responses to these supplements untrustworthy as either a diagnostic or therapeutic approach. Pastures can be top-dressed with copper containing salts (Gallagher and Cottrill, 1985; Smart et al., 1992; Suttle, 1986 a). Parental injection or intraruminal copper oxide needles can treat individual animals. All these approaches have been used with moderate degrees of success (Rogers and Poole, 1988; Larson et al., 1995; Suttle, 1986 b).

THE ROLE OF COPPER STATUS IN THE IMMUNE RESPONSE IN OTHER SPECIES

Copper has a wide range of activities relative to the host immune response. Ceruloplasmin, a copper containing enzyme, is an acute phase protein. During inflammation hepatic production increases dramatically in response to the increased need to scavenge oxygen radicals released by immune cells (Percival, 1998). In copper deficient male rats there is a decrease in protection from oxidative damage that may impair supply of oxygen to tissues (Fields et al., 1996). Copper-zinc superoxide dismutase (SOD) functions to eliminate oxygen radicals to oxygen and hydrogen peroxide. The copper containing portion of this molecule functions as the superoxide-binding site. Studies have now shown that SOD is critical to antioxidant defense. Transgenic mice with high SOD activity have an increased resistance to reperfusion injury induced by leukocytes and macrophages (Percival, 1998). In rats fed a copper deficient diet, it was evident that SOD activity is closely linked to the overall copper deficiency (Bohnenkamp and Weser, 1976). Respiratory burst is essential in the normal function of tissue macrophages, specifically cytosolic damage to invading micro-organisms. It is hypothesized that copper supplementation increases the respiratory burst of activated liver macrophages (Sans et al., 1999). In male rats fed a diet of marginally low copper diet decreases in immune cell function were noted. Specifically splenic T lymphocytes were less responsive to in vitro mitogenic activation and generated less interleukin-2 bioactivity than did cultures from rats' fed a copper replete diet. Neutrophils

from rats fed the low copper diet generated superoxide anion that was 60% of those fed the replete diet (Hopkins and Failla, 1995).

Cytochrome oxidase *C* is the terminal enzyme in membrane respiration transport chain. In severe copper deficient states the respiratory capacity of mitochondria in the heart, liver and brain is reduced. Metallothionines are small polypeptides responsible for storage and detoxification of metal ions that otherwise might interfere with normal homeostasis. These peptides can bind many metal ions; however, only zinc, copper and cadmium binding is significant in vivo. Copper metallothionines may function in copper transport to donate copper ions to enzymatic systems. Recent evidence suggests that they may function in antioxidant defense by scavenging hydroxyl and superoxide radicals (Linder and Hazegh-Azam, 1996). In adult, healthy, male humans fed a copper deficient diet there was a significant decrease in the proliferation of peripheral blood mononuclear cells. But this had no measurable effect on the immune function of the various cell types including neutrophils (Kelley et al., 1995).

Several functions of the immune system require copper. Less is known about the mechanisms of action in some of these functions. Copper deficiency reduces the effectiveness of the acquired immune response. IL-1 production is increased, and IL-2 production is decreased. Proliferation of lymphocytes in response to mitogen stimulation in copper deficient mice is decreased (Percival, 1998). In addition to the decrease in IL-2 production; copper deficiency in rodents decreases the numbers of T-helper (CD4+) and total T cells. This impairment of cell proliferation is reversible by both in vitro and in vivo copper supplementation (Bala and Failla, 1993; O'Dell, 1993). Effects of this decrease in T-helper cells may impair antibody response to certain mitogens. In macular

mutant mice, a model for Menkes' kinky hair disease, antibody response to sheep red blood cells and dinitrophenyl-ficoll was suppressed when compared to genetically normal mice (Nakagawa et al., 1993). Alterations in lymphocyte cell membrane protein and lipid composition have been found in copper deficient mice. These changes are thought to influence membrane fluidity and host immune response (Korte and Prohaska, 1987). The protein structure of red cell membranes are also altered in copper deficient rats. Although this may affect the function of the red cell, it may be more useful as monitoring the course of disease or its treatment (Miller et al., 1995). In humans and mice neutropenia is a hallmark of copper deficient states (Hayton et al., 1995; Tamura et al., 1994; Wasa et al., 1994). Bone marrow aspirates from copper deficient individuals show maturation arrest. Anti-neutrophil antibodies have been detected in copper deficient patients. These neutrophils have impaired function including decreases in superoxide anion production and decreased candidacidal activity without reduction of phagocytosis. Even marginal copper deficiency impairs neutrophil function. Expression of the adhesion molecule CD11b is reduced on neutrophils from copper deficient subjects (Percival, 1998).

THE ROLE OF COPPER STATUS IN THE IMMUNE RESPONSE IN CATTLE

Less research regarding the effects of copper deficiency on immune function in cattle has been performed. Copper deficiency seems to have little effect on lymphocyte blastogenesis in cattle (Ward et al., 1997). Calves born to dams fed a copper deficient diet have increased levels of tumor necrosis factor and other cytokines (Gengalbach, 1997). In copper deficient heifers challenged by bovine herpes virus-1, fibrinogen, a non-copper containing acute phase protein was increased 48 hours after exposure. In this study no

change in superoxide dismutase activity or lymphocyte proliferation was detected. Unfortunately there were only 6 animals in the study group and 6 in the control (Arthington et al., 1996). This makes interpretation of negative results difficult without a calculation of power. Neutrophilia and increases of fibrinogen concentration have been observed in copper deficient calves. Lymphocyte blastogenesis appeared unchanged. Phagocytosis of *Staphylococcus aureus* was not affected, but bacteriocidal activity may be decreased (Arthington et al., 1996). A recent study in 6 female cattle fed a copper deficient diet showed a significant decrease in B-lymphocytes and reduced neutrophil activity. The authors interpreted this as a possible contributor to a greater incidence of infectious diseases in copper deficient cattle (Cerone et al., 1998). In addition; Gengelbach and Spears found that calves fed a molybdenum supplemented diet in order to induce copper deficiency had decreased SOD activity and serum antibody response to porcine erythrocytes (Gengelbach and Spears, 1998).

In these studies copper deficiency was induced by either molybdenum or iron supplementation. This complicates interpretation of results. Although calves were clearly copper deficient based on liver copper concentrations, (Ward et al., 1997) it is unclear whether the results were a consequence of excess molybdenum or iron, or alternatively due to a deficiency in copper.

In one recent study comparing steers grazing endophyte infected tall fescue and steers grazing non-endophyte infected fescue some interesting observations have been made. Those calves grazing endophyte-infected fescue had low serum copper and ceruloplasmin concentrations. A decreased monocyte phagocytic activity and expression of major histocompatibility class II antigens was observed (Saker et al., 1998). Although

it is unclear from this study whether the compromised immune function is due to the copper deficiency or to the endophyte intoxication, this model resembles husbandry in much of the United States. Tall fescue is common forage in many areas of the United States (Corah and Dargatz, 1999).

ROLE OF COPPER DEFICIENCY AND MATRIX METALLOPROTEINASES IN LUNG INJURY

There is a well accepted association between copper deficiency and acute respiratory distress syndrome (ARDS) in human patients (Ho et al., 1998). This seems contrary to the pathophysiological consequences of copper deficiency. Copper deficiency is known to adversely effect neutrophil development and function (Percival, 1998). With the knowledge that a large portion of lung injury in ARDS is due to the influx of neutrophils, (Jernigan et al., 2004), one would conclude that copper deficiency should have a protective effect in these patients.

Recently Lentsch et al, believe they have found an explanation for the clinical observation of increased lung injury with copper deficiency. In a model of acute lung injury in rats, investigators found a significant increase of MMP-2 and MMP-9 in copper deficient versus copper replete mice. Enhanced lung injury in this model was attributed to this augmented inflammatory cell independent MMP production. The augmented MMP levels identified were unrelated to alveolar macrophage or neutrophil cell populations. The investigators concluded that the increased MMPs were a result of lung epithelial and/or endothelial cell stimulation (Lentsch et al., 2001).

CHAPTER 2

SENSITIVITY AND SPECIFICITY OF SERUM COPPER DETERMINATION FOR DETECTION OF COPPER DEFICIENCY IN FEEDER CALVES

INTRODUCTION

Copper is an essential component of several enzymes that are required to maintain host homeostasis (Radostitis et al., 1994). Copper deficiency has been linked to a variety of clinical signs, including pale coat, poor fleece quality, anemia, spontaneous fractures, poor capillary integrity, myocardial degeneration, hypomyelinization of the spinal cord, impaired reproductive performance, decreased resistance to infectious disease, diarrhea, and generalized ill-thrift (Radostitis et al., 1994; Larson et al., 1995; Wikse et al., 1992). None of these clinical signs are pathognomonic for copper deficiency, which is readily confused with other diseases. Consequently, many copper-deficient animals are unrecognized as such (Radostitis et al., 1994; Larson et al., 1995).

The liver is the primary copper storage organ (Radostitis et al., 1994; Gooneratne et al., 1989; Brewer, 1987). Copper is released from liver stores to maintain blood copper concentrations and essential physiologic functions (Radostitis et al., 1994; Larson et al., 1995; Wikse et al., 1992). Consequently, serum copper concentration may overestimate total body copper stores during copper deficiency and underestimate body copper stores during copper toxicosis. During copper toxicosis, intake exceeds demand (Radostitis et al., 1994; Larson et al., 1995; Wikse et al., 1992). Liver storage far exceeds mobilization,

resulting in net hepatic accumulation of copper (Radostitis et al., 1994; Larson et al., 1995; Wikse et al., 1992); however, serum copper concentration is maintained within a narrow reference range. When liver stores reach a critical threshold, acute hepatic necrosis develops, releasing liver copper stores and causing transient high serum copper concentration. As a direct consequence, serum copper determinations will typically not permit diagnosis of copper toxicosis unless sampling is coincidental with acute hepatic disease (Radostitis et al., 1994; Kumaratilake and Howell, 1989). If ruminants are given copper-deficient diets, liver stores will be mobilized, maintaining serum copper concentrations within reference range and supporting vital functions. These patterns of storage, mobilization, and utilization directly impact our interpretation of serum copper concentrations. High serum copper concentration is suggestive of copper toxicosis with coincidental release of liver copper. Serum copper concentration within reference range is consistent with high liver copper stores without coincidental liver copper release, normal liver copper stores, or low liver copper stores that are being mobilized to maintain serum copper concentration. Low serum copper concentration suggests that liver copper stores have been exhausted and the animal is in a deficient state.

A recent survey conducted by the National Animal Health Monitoring Service classified 14.2% of US forage samples as copper deficient and 49.7% of forage samples as having marginal copper content. High molybdenum and iron concentrations also were observed commonly in these forage samples (Corah and Dargatz, 1996). High iron and molybdenum concentrations will impair copper utilization (Gengelbach et al., 1997; Campbell et al., 1974; Suttle, 1991; Ward et al., 1993). This propensity for copper

deficiency was substantiated by results of a recent survey that classified 40.6 % of US beef cattle as copper deficient (Dargatz et al., 1999). It should be noted that this study based the diagnosis of copper deficiency on a serum copper concentration of < 0.65 μg/g. Additionally, this survey studied cows without regard to age or pregnancy status (Dargatz et al., 1999).

A definitive serum copper concentration that reflects decreased liver copper concentration has not been established. Reference ranges and threshold concentrations have been presented in texts and survey reports; however, these values vary greatly and often provide references ranges for serum copper concentrations for deficient and replete cattle which overlap (Radostitis et al., 1994; Dargatz et al., 1999; Puls, 1994; Maynard et al., 1979; Howell and Gawthorne, 1987). Consequently, diagnosis of copper deficiency on the basis of serum copper concentration is problematic. Authors of previous studies (Suttle, 1986 a; Suttle, 1986 b) often have discounted the value of serum copper determinations in the diagnosis of copper deficiency. The purpose of the study reported here was to determine the relationship between serum and liver copper concentrations in juvenile beef calves and evaluate serum copper determination for diagnosis of copper deficiency.

MATERIALS AND METHODS

Calves - Paired liver and serum samples were obtained from 105 calves that were 6 to 9 months old. Thirty-three of these calves were randomly chosen from a group of recently weaned calves fed grass pasture ad libitum 28 days prior to the study. These calves

received neither grain nor mineral supplementation. A second group consisted of 36 recently weaned calves that had been purchased at a northern Missouri sale barn. These calves were fed a total mixed ration containing hay, grain, and a mineral supplement for 7 days prior to the study. A third group consisted of 36 calves fed the aforementioned total mixed ration for 28 days prior to the study. The wide range of diets and sources of these calves ensured that calves would have highly variable copper status. Variability in serum copper content of our samples may also have been increased by stressful events such as shipping, which may cause increased concentrations of the acute phase protein ceruloplasmin.

Liver and serum sample collection - Serum samples were obtained by venipuncture of the coccygeal or jugular vein. Blood was collected into evacuated tubes with royal blue rubber stoppers that are produced without zinc to avoid interference with trace mineral analysis. Serum was harvested after clot formation and centrifugation and placed in sterile plastic containers (Tube, self-stand graduated with caps. Denville Scientific, Inc., 3005 Havley Road, South Plainfield, NJ). Liver samples were obtained by use of a transthoracic technique (Saker et al., 1998; Pearce et al., 1997), using a 16-gauge soft tissue biopsy needle (16 gauge X 16 cm (J-118c), 1 mm X 20 mm specimen chamber. Jorgensen Laboratories Inc, Loveland, Colo). Three or 4 liver biopsy samples were obtained from each calf, placed in sterile plastic containers, and transported on ice in a cooler. All samples were frozen until analysis was performed.

Serum and liver copper analysis - Serum and liver samples were analyzed by use of atomic absorption spectrophotometry (Perkin-Elmer 2380 Atomic Absorption Spectrophotometer, Perkin-Elmer, Norwalk, Conn.) (wavelength, 324.7 nm). Serum copper determination was performed with external controls containing 1.99 μg/g copper, and copper standards of 1, 0.5, 0.2, and 0.1 μg/g were prepared by use of calibration reference solution (Copper Reference Solution 1,000 ppm ± 1%, Fischer Scientific Company, Fair Lawn, NJ.) and 0.5% Triton X-100 (Triton X-100, Fischer Scientific Company, Fair Lawn, NJ.). A standard curve was generated via regression analysis of the copper standards. One milliliter of serum was added to 1 ml of 0.5 % Triton X-100 in a plastic tube and vortexed before analysis. Copper concentrations of individual samples were determined by comparison to the standard curve. All concentrations were measured in micrograms per gram wet weight.

Liver samples were placed in a tared Falcon tube (Falcon tube 15 ml conical, Fischer Scientific Company, Fair Lawn, NJ.). Sample wet weight was recorded, and 0.5 ml of concentrated HNO_3 (Nitric Acid TraceMetal Grade, 68 – 71%, Fischer Scientific Company, Fair Lawn, NJ.) was added to the vial. The vial was capped and the sample was digested for 12 hours in an oven at 95 C. The sample solution was diluted to a final volume of 2 ml. Copper standards of 0.1, 0.25, 0.5, 1.0, and 2.0 μg/g were prepared as described. Samples were analyzed by group with separate standard curve determinations for each group. Standard curve equations were produced by use of regression analysis. Final liver copper concentration calculations were determined by comparison with respective standard curves and division by starting wet weight. Analysis techniques were

similar to the methods published by the Association of Official Analytical Chemists (Osheim, 1983; Osheim and Ross, 1985), with the exception of substituting 0.5% Triton X-100 (Triton X-100, Fischer Scientific Company, Fair Lawn, NJ.) for deionized distilled water.

Data analyses - Initially, simple linear and polynomial regression models were developed that predicted liver copper concentrations as a function of serum copper concentrations. Calculations were performed by use of a statistical computer software package (Sigma-Stat version 2.03, Jandel Scientific Software, San Rafael, Calif.).

Additionally, standard methods to determine the sensitivity and specificity of serum copper concentration for detection of low liver copper concentrations were used. For all calculations, adequate copper status was defined as a liver copper concentration ≥ 25.0 μg/g wet weight as determined by use of atomic absorption spectrophotometry. Copper deficiency was defined as liver copper concentration < 25.0 μg/g wet weight (Puls, 1994). Sensitivity and specificity of serum copper concentration were calculated, using test endpoints of ≤ 0.40, 0.45, 0.50, 0.55, 0.60, 0.65, 0.70, 0.75, 0.80, 0.85, 0.90, 0.95, 1.0, and 1.5 μg/g wet weight of serum as determined by use of atomic absorption spectrophotometry. Additionally, true positive, true negative, false positive and false negative rates for each of these endpoints were determined (Fig 1). Sensitivity was defined as the number of calves correctly identified as copper deficient (true positives) divided by the number of all copper-deficient calves (true positives plus false negatives) identified at each test endpoint. Specificity was defined as the number of calves correctly

identified as copper replete (true negatives) divided by the number of all copper-replete calves (true negatives plus false positives) identified at each test endpoint. A test endpoint that maximized sensitivity and specificity was chosen. These formulae are described elsewhere (Tyler and Cullor., 1989) in greater detail. By use of the chosen test endpoint and accompanying sensitivity and specificity, real prevalence was calculated for all possible apparent prevalences by use of the equation:

Real prevalence = (Apparent prevalence + Specificity – 100%) / (Sensitivity + Specificity – 100%) (Smith, 1995).

By use of the chosen test endpoint, positive and negative predictive values were calculated for all possible prevalences by use of the equations:

Negative predictive value = (Prevalence X [1 – Sensitivity]) / ([Prevalence X {1 – Sensitivity}] + [{1 – Prevalence} X {Specificity}]); and

Positive predictive value = (Prevalence X Sensitivity) / (Prevalence X Sensitivity + [{1- Prevalence} X {1- Specificity}]).

Significance was set at $P < 0.05$.

RESULTS

Mean **(±** SD**)** liver copper concentration was 75.85 ± 63.55 μg/g (range, 4.89 to 231.67 μg/g). Thirty-two of the 105 liver samples had copper concentration ≤ 25.0 μg/g. Mean serum copper concentration was 0.64 ± 0.23 μg/g (range, 0.18 to 1.32 μg/g). Results of linear regression ($P < 0.001$) and polynomial regression ($P = 0.01$; (Fig 2) models indicated significant associations between liver and serum copper concentrations.

A test endpoint for serum copper concentration of $\leq$ 0.45 μg/g optimized test sensitivity (0.53) and specificity (0.89; (Table 1). Real prevalences for all possible apparent prevalences were calculated by use of the aforementioned sensitivity and specificity values (Fig 3). Predictive values of positive and negative tests at all possible prevalences were determined (Fig 4).

DISCUSSION

The study population described here was chosen to satisfy the guidelines presented by Sackett et al (Sackett et al., 1991) for evaluation of a diagnostic test. Specifically, the guideline that the study population must be representative of the spectrum of disease, yet be fairly uniform, was considered crucial. In previous studies (Vermunt and West, 1994; Claypool et al., 1975) that attempted to define the relationship between serum or plasma copper concentrations and liver copper concentrations, study populations had large variations in age distribution. Our study focused on those calves at greatest risk for detrimental effects attributable to copper deficiency. Calves are predisposed to copper deficiency because their primary nutritional support derives from copper-deficient milk or pastures (Bingley and Dufty, 1972). Calves that were supplemented with a total mixed ration (n = 36) were included to ensure that some calves were copper replete. A previous experimental study (Dargatz et al., 1999) included suckling calves, weaned calves, replacement heifers, feedlot steers, and adult cows. Additionally, many dairy cattle were included. These cattle would have dramatically reduced risk for copper deficiency.

Furthermore, most dairy cows are fed either a total mixed ration or an individual grain supplement, both of which contain micronutrient mineral supplementation.

Although a significant association was observed between serum copper concentration and liver copper concentration in the 2 regression models, the strength of these associations was inadequate to permit accurate prediction of actual copper status. This result was consistent with results of previous studies (Vermunt and West, 1994; Claypool et al., 1975).

It follows that an alternative means of defining this relationship should be used to predict calf liver copper concentration. The obvious choice was to calculate the sensitivity and specificity of serum copper concentration as a predictor of liver copper concentration at several test endpoints. The accepted pattern of copper homeostasis is one in which liver copper stores are mobilized to maintain serum copper concentrations. This model suggests that a threshold rather than a direct linear relationship should exist between serum and liver copper concentrations. This type of relationship is well suited to a traditional epidemiologic approach in which test results are classified as positive or negative. Claypool et al (Claypool et al., 1975) suggested a plasma copper concentration of < 0.50 μg/g as indicative of copper deficiency. Vermunt and West (Vermunt and West, 1994) identified a similar value for diagnosis of copper deficiency when using serum. Our optimal endpoint serum copper concentration of 0.45 μg/g was similar. Unfortunately, in neither study was test sensitivity, specificity, nor predictive values reported. To the authors' knowledge, the study reported here is the first to determine

sensitivity and specificity of serum copper determinations for detection of low liver copper concentration.

No attempt was made to distinguish between severe and marginal copper deficiency. We defined abnormal calves as having liver copper concentration < 25 μg/g wet weight, thus including historically defined marginally and severely deficient calves within 1 group. As other authors (Corah and Dargatz, 1996) have stated, subclinical copper deficiency is apparently widespread in the United States and is probably more important in terms of economic losses than is clinical copper deficiency.

At the chosen test endpoint (serum copper concentration, 0.45 μg/g), sensitivity and specificity were 0.53 and 0.89, respectively. Increasing the test endpoint increased test sensitivity, but specificity decreased precipitously. A higher endpoint would more readily identify copper-deficient calves, but would false-positive identifications would increase. False-positive test results may have negative economic impact, because unnecessary copper supplementation would probably not enhance productivity. With these economic constraints in mind, it behooves us to be confident that calves classified as copper deficient, truly are copper deficient. Although calves that are not copper deficient would not benefit from copper supplementation, they probably would not develop adverse effects. Feeder calves that are even marginally copper deficient would benefit greatly from supplementation. For this reason, we chose a test endpoint for serum copper concentration of $\leq$ 0.45 μg/g. Results of testing individual calves may be misleading because of the low sensitivity, but if numerous calves in a herd were tested and even a

few were identified as copper deficient, the high specificity of the test would provide confidence that the herd in general was copper deficient.

Given the sensitivity and specificity of serum copper determinations for detection of copper deficiency that we determined, we may draw conclusions regarding how this test will perform as a diagnostic test. In a population with no copper deficiency (0.0% prevalence), 11% of tested calves will have positive test results (serum copper concentration, ≤ 0.45 μg/g). In a population in which all calves are copper deficient (100% prevalence), 53% of calves will have positive test results. Thus, the percentage of positive test results will vary from 11 to 53%.

Before we discount the potential value of serum copper determination as a diagnostic procedure, it behooves us to examine the performance of this procedure in the perspective of other common diagnostic tests. For example, many states advocate the use of serologic testing to identify cattle infected with *Mycobacterium paratuberculosis* (Johne's disease). In stage I of this disease (early preclinical Johne's disease), reported test sensitivity is 24.6 %. In stage II (late preclinical Johne's disease) reported sensitivity is 56.5 % (Collins and Socket, 1993). One could readily argue that the measurement of serum copper concentration yields equivalent or superior results, compared with this accepted test procedure. Furthermore, the negative consequences of a false-positive result for a test for copper deficiency are clearly of less consequence than the consequences of a false-positive result for a test for Johne's disease. A calf with a false-positive diagnosis of

copper deficiency will be provided supplemental copper, whereas a calf with false-positive diagnosis of Johne's disease will likely be targeted for culling.

The applications of serum copper determinations in a herd setting are readily apparent. Apparent prevalence of copper deficiency, obtained by analysis of representative samples, may be used to calculate an estimate of true prevalence. If the estimated prevalence is low, further diagnostic procedures are probably not indicated, particularly given the satisfactory predictive value of a negative test at low prevalences. If the calculated true prevalence is high, further diagnostic testing, including liver copper determination, is probably indicated.

At prevalences < 20%, apparent prevalence overestimates true prevalence. At higher prevalences, apparent prevalence underestimates true prevalence, and this finding is accentuated as prevalence increases. Inspection of predictive values at all possible prevalences revealed that the predictive value of negative test results was acceptable at even a 20% prevalence of copper deficiency. Predictive values of a positive test result are unacceptably low in populations with low prevalence of copper deficiency, but increase as prevalence increases. This indicates that negative test results are meaningful as a means with which to rule out a differential diagnosis of copper deficiency, but positive test results will not confirm a diagnosis of copper deficiency. Interpretation of serum copper concentrations in individual calves remains problematic.

Optimal diagnosis of copper deficiency presently requires determination of liver copper concentration, yet many practitioners and producers are reluctant to perform liver biopsies. Thus, serum copper determination may provide a useful approach to rule out copper deficiency in cattle with suboptimal performance. Knowledge of the test's sensitivity and specificity should permit improved assessments of herd copper status.

CHAPTER 3

FACTORS RELATED TO COPPER STATUS IN SPRING-BORN MISSOURI FEEDER CALVES

INTRODUCTION

Copper is an essential micronutrient (Larson et al., 1995; Smart et al., 1992). Copper deficiency has been associated with disease states that decrease commercial beef production. Clinical manifestations of copper disease include anemia, diarrhea, long bone fractures, generalized ill-thrift, and decreased fertility (Smart et al., 1992; Gay et al., 1988; Goonratne et al., 1989).

Recent studies have demonstrated that copper deficiency is common in North American beef cattle (Smart et al., 1992; Dargatz et al., 1999). Dargatz et al. found that 40.6% of the beef cows and heifers were either deficient or marginally deficient. This study demonstrated that copper deficiency was common even though half of the producers reported using a copper supplement. It should be noted that Dargatz et al. used a test endpoint of less than 0.65 μg/g serum copper concentrations to define marginal deficiency. Results of a recent study suggested that this test endpoint might have resulted in wholesale misclassification of copper replete calves as copper deficient (Tessman et al., 2001). A test endpoint of less than or equal to 0.45 μg/g best optimizes test performance.

The primary purpose of this study was to identify management factors that would affect serum copper concentration and copper deficiency. In addition, we wished to determine whether copper status was related to owner perceptions regarding the occurrence of disease. Although in this study only herds from Missouri were surveyed, the conclusions reached

should be applicable in other areas. General management practices for beef herds are similar across the Midwest and the vast majority of Missouri calves are fed in other states.

MATERIALS AND METHODS

Sample and survey data collection - The data collection process was a systematic attempt to determine the copper status of feeder calves throughout Missouri. The sampling strategy was premised upon geographic localities (counties), rather than proportionate sampling of cattle populations. Private veterinary practitioner-collaborators whose practice included a large beef cattle component were identified throughout the state. Collaborator veterinarians collected blood samples from 3 representative calves in each enrolled herd and obtained samples from no more than 3 herds in each county. Sampling was performed at the time of routine fall processing of calves and was restricted to calves between the ages of 4 and 10 months. Practitioners completed a questionnaire summarizing exposure to potential risk factors for copper deficiency. The survey included questions regarding region, calf age in months, pasture type and mineral supplementation practices. Owners also were asked whether diarrhea of mature cows, calf diarrhea, pneumonia, fractures, abnormal hair coats, lameness and cow fertility were perceived as ongoing health problems in their herd.

Serum copper analysis - Serum samples were analyzed by use of atomic absorption spectrophotometry (Perkin Elmer 2380 Atomic Absorption Spectrophotometer, Perkin Elmer, Norwalk, CT) (wavelength, 324.7 nm). Serum copper determination was performed with external controls containing 1.99 μg/g copper, and copper standards of 1, 0.5, 0.2, and 0.1 μg/g were prepared by use of calibration reference solution (Fischer Scientific Company, Fair

Lawn, NJ) and 0.5% Triton X-100 (Fischer Scientific Company, Fair Lawn, NJ). A standard curve was generated via regression analysis of the copper standards. One milliliter of serum was added to 1 ml of 0.5 % Triton X-100 in a plastic tube and processed through a vortex before analysis. Copper concentrations of individual samples were determined by comparison to the standard curve.

Data analysis - Low serum copper concentration was defined as a serum copper concentration ≤ 0.45 μg/g wet weight (Tessman et al., 2001). Adequate serum copper concentrations were defined as > 0.45 μg/g wet weight. Only calves for which serum copper concentration and complete survey data were obtained were included. Apparent prevalence, (the proportion of calves with serum copper concentrations ≤ 0.45 μg/g) was reported for each of the 9 agricultural districts (Northeast, North Central, Northwest, West Central, Central, East Central, Southeast, South Central and Southwest) as defined by the Missouri Agriculture Statistics Service (Missouri Department of Agriculture, Jefferson City, MO). Additionally, real prevalence was defined using the following equation: $RP= (AP + S_p – 1) / (S_e + S_p – 1)$ (Martin, 1984), where RP is real prevalence, AP is apparent prevalence, S_e is sensitivity and S_p is specificity. Values used for sensitivity and specificity, 53% and 89%, respectively, in the calculation were drawn from a previously study (Tessman et al., 2001). Proportions were compared among districts using a 2-by-9 chi-squared test (The SAS System for Windows, Version 8, SAS Institute Inc., Cary, North Carolina). Patterns of deficiency were deemed to differ when the calculated P value < 0.05. To calculate the statewide proportions of calves with deficient copper status, we multiplied the proportion of calves with low serum copper concentrations in each region times the number of calves in the respective region to get the number of calves in each region with less than optimal copper status. These numbers then were

summed and divided by the total state beef calf population, yielding statewide weighted proportions.

Associations between owner perceptions of herd health and calf serum copper concentrations were explored using a series of chi-squared tests. Observations were cross-classified using 2-by-2 tables defined by disease status (0, 1) and serum copper concentrations (≤ 0.45 μg/g). Disease status variables included the presence of diarrhea in mature cows, calf diarrhea, pneumonia, fractures, abnormal hair coats, lameness and cow fertility.

Forward stepwise logistic regression models were developed to predict the incidence of low serum copper status (≤ 0.45 μg/g) as a function of region, calf breed, calf age, animal health and husbandry practices, pasture type and mineral supplementation practices. In each regression model, the independent variable with the smallest P-to-enter was added at each step until no remaining variable had a P-to-enter > 0.05. Calculations were performed with the aid of a statistical software package (SAS Institute Inc., Cary, NC).

RESULTS

Of the 528 calves studied, 34% had access to creep feed and 71% of the calves had access to trace mineralized salt. Eleven percent (11%) of the calves originated from herds provided with supplemental hay and 16% of the calves originated from herds with supplemental concentrates. Many of the operations fertilized pastures (84%). Fifty-eight percent of the operations utilized only commercial fertilizer, 5% used only manure from various species, and 21% used both. The predominant pasture plant was fescue (94% of pastures); however, orchard grass (31%) and red clover (44%) were common. More detailed description of the study population is available upon request.

Serum copper concentrations varied from 0.06 to 2.25 μg/g. Eighteen percent (18%) or 96 of the total number of calves sampled had serum copper concentrations less than or equal to 0.45 μg/g (Figure 5). Apparent prevalence of low serum copper concentration (≤ 0.45 μg/g wet weight) varied from 4% to 33% among the 9 agricultural districts. The calculated real prevalence of copper-deficient calves varied from 0 to 53% by agricultural district. The highest proportion of deficient calves was observed in the Southeast district. Two districts, Southwest and South Central, were defined as zero calculated real prevalence because the actual value calculated was less than zero. The calculated statewide real prevalence of copper deficiency was 17.1% (Table 2). The proportion of copper-deficient calves differed among the 9 agricultural districts ($p< 0.05$). Low serum copper concentrations (≤ 0.45 μg/g) were not significantly associated with owner perceptions that cow diarrhea, poor hair coats, infertility, calf diarrhea, pneumonia, fractures or lameness were problems in the herd.

The regression model predicting serum copper concentration revealed a large number of associations between serum copper concentration and independent variables (Table 3). The Southwest and South Central regions, calves more than 6 months of age, lespedeza pastures, creep feed and trace mineralized salt were associated with an increase in serum copper concentration. The West and East Central regions, poor cow condition, and white clover pastures were associated with a decrease in serum copper concentration.

The logistic regression model predicting copper deficient status identified several significant associations with postulated independent variables (Table 4). Ladino clover pastures were associated with copper-deficient status. The Southwest and South Central

regions, alfalfa pastures, and providing creep feed and trace mineralized salt were associated with a decreased likelihood of copper-deficient status.

DISCUSSION

Copper deficiency, (≤ 0.45 μg/g), was found in 7 of the 9 agricultural districts. Two districts, Southwest and South Central, with no copper deficiency are located in the Ozark Plateau, a region of relatively higher elevation. We estimated that more than 300,000 calves were copper deficient. Inspection of a map of Missouri indicates that the patterns of most severe copper deficiency were observed in the Missouri and Mississippi river basins (Figure 6). This observation agrees with the observation that river silt pastures may be associated with trace element deficiencies (Radostitis et al., 2000). Explanation of this phenomenon may lay in a recent report describing the complex interaction of trace elements, most notably zinc and copper, with dissolved organic matter in fresh water (Rozan et al., 2000). Highly stable complexes that are resistant to disassociation are formed.

Although fescue pastures were the most common forage provided across the state (94%), this was not significantly associated with copper status or serum copper concentration. This is contrary to recent reports of endophyte-infected fescue being associated with decreased available copper (Dennis et al., 1998). The survey made no attempt to differentiate endophyte versus non-endophyte infected fescue. In addition the small number of operations (31 herds) that did not utilize fescue limits the ability to detect differences.

Pasture types that were significantly associated with serum copper concentration include lezpedeza (increased concentration) and white clover pastures (decreased concentration). Pasture types significantly associated with copper status are ladino clover pasture (positive) and alfalfa (negative). In general, legumes tend to be higher in copper concentration than grasses (Minson, 1990). This observation presents a conundrum as all of these pasture types are legume-type forages. It is possible that these disparities are the result of random chance. Given the strength of the associations (Tables 3 and 4), this seems unlikely.

Not surprisingly, access to creep feed and trace mineralized salt were positively associated with serum copper concentration and negatively associated with the probability of copper deficiency. Both of these practices are recommended as therapeutic preventatives for copper deficiency (Larson et al., 1995; Radostitis et al., 2000).

Older calves had increased serum copper concentrations and calves in herds with poor cow body condition had decreased serum copper concentrations. Both of these relationships are intuitively logical. Older calves will rely more heavily on pasture forage and concentrates. Therefore, the risk of copper deficiency associated with a cow's milk diet will be decreased (Naveh et al., 1981; Jamieson and Allcroft, 1950). In the instance of cows with poor body condition, one can intimate that this is a reflection of overall herd management. The fact that the cows themselves are in poor condition suggests an overall lack of good quality available feedstuffs. This lack of quality feed easily could lead to a copper deficiency as well as deficiencies in other micronutrients.

None of the health-related questions in the survey are significantly associated with copper deficiency in this study. This is of particular interest as most of the questions

posed pertain to disease syndromes that have been historically linked to copper deficiency (Larson et al., 1995; Smart et al., 1992; Radostitis et al., 2000). It is possible that there were too few of those syndromes observed to make a statistically significant association. Other explanations may include a lack of owner awareness to the various disease entities.

CHAPTER 4

USE OF A BINOMIAL MODEL TO PREDICT A LOWER CONFIDENCE LIMIT FOR COPPER DEFICIENCY PREVALENCE IN FEEDER CALVES

INTRODUCTION

Ideally, diagnostic tests have high sensitivity and specificity. When tests are used to direct the medical management of individuals the accuracy of individual test results is paramount. Additionally researchers, clinicians and public health professionals are often asked to provide estimates of disease prevalence based on results of imperfect tests. Reasonable conclusions regarding the population disease behavior may be made using tests with less than optimal sensitivity and specificity. Traditionally real prevalence is calculated using the following formula:

Equation (Eq) 1: $RP = (AP + S_p - 1) / (S_e + S_p - 1)$. (Martin, 1984)

Where RP is real prevalence, AP is the apparent prevalence or proportion of positive test results, S_p is the specificity or likelihood of a negative test in a disease negative individual and S_e is the sensitivity or likelihood of a positive test in a disease positive individual. It is possible to use the Z-distribution for population proportions to construct lower confidence intervals for calculated real prevalence using the following formula:

95% lower confidence limit of prevalence = $RP - 1.96 \sqrt{(RP(1-RP)/n)}$, (Daniel, 1999)

where, RP = calculated real prevalence, and n = sample size. It should be noted that small sample sizes produce broad confidence intervals, which have little value in describing population prevalence.

The purpose of this study was to develop an alternate approach based on the binomial probability distribution. For illustrative purposes we examined detection of copper deficiency in cattle using serum copper concentrations. This procedure has previously been reported to have imperfect sensitivity (0.53) and specificity (0.89)(Tessman et al., 2001). True copper status was determined by liver copper concentrations.

MATERIALS AND METHODS

Theoretical reasoning - The hypothesis statement developed to guide model development was as follows:

H_0: Real prevalence ≤ an a priori hypothesized prevalence

H_a: Real prevalence > an a priori hypothesized prevalence

It is intuitive that apparent prevalence equals the probability of a positive test result, AP = Pr{T+} (Martin, 1977). Eq 1 can be solved for apparent prevalence to obtain equation 2,

Equation (Eq) 2: $AP = RP*S_e + RP*S_p - RP - S_p + 1$.

The binomial distribution requires an outcome, which is either positive or negative and mutually exclusive; Pr{T+} = 1 - Pr{T-}, where Pr{T+} is the probability of a positive test and Pr{T-} is the probability of a negative test. The reported sensitivity and specificity were used to calculate the Pr{T+} at all possible prevalences. These

probabilities were used with various combinations of trials and successes in the binomial distribution equation to calculate the probability of k positive test results (serum copper ≤ 0.45 μg/ml) when n subjects are sampled at varying prevalences. The binomial distribution may be defined as follows,

Equation (Eq) 3: $P(X=k) = \binom{n}{k} p^k (1-p)^{n-k}$. (Moore and McCabe, 1993)

Where P(X=k) is the probability of an event, n is the number of trials, k is the number of positive outcomes and p is the probability of a success in each trial. In our example, n was the number of calves sampled, k was the number of animals with a serum copper concentration less than or equal to 0.45 μg/g, and p was the hypothesized apparent prevalence as calculated using Eq 2. The event predicted was the likelihood of the number of positive tests being greater than or equal to k when n calves were sampled in a population of defined prevalence. If the calculated probability was very low, we assumed that the prevalence is higher than that which was previously hypothesized. The probability statement may be expressed as, $Pr\{RP \geq x\} \leq$ p-value. Because this model was based on apparent prevalence, an additional probability statement was required. This statement may be expressed in the form of $Pr\{AP \geq y\} \leq$ p-value. Therefore, the final probability statement required to reject hypotheses regarding prevalence based on randomly sampled data sets was as follows

Equation (Eq) 4: $Pr\{RP \geq x\} = Pr\{AP \geq y\} = \Sigma \binom{n}{k} p^k (1-p)^{n-k} \leq$ p-value.

When the calculated $Pr\{AP \geq y\} \leq 0.025$ we rejected the null hypothesis that real prevalence is less than an a priori hypothesized prevalence. We chose a p-value ≤ 0.025 because it is equivalent to the lower limit of a 95% confidence interval.

With the aid of a computer program (Microsoft Excel 2000, Seattle, WA) a large table representing the probability of all possible outcomes for trials consisting of greater than or equal to 5 and less than or equal to 20 samples at all possible real prevalences was produced.

Model validation - Serum copper determinations were performed on samples taken from calves ranging in age from 6 to 9 months from a single herd. Paired liver and serum samples were collected from 33 calves. Blood was collected into evacuated tubes specifically manufactured for trace mineral determinations (Becton Dickinson and Company, Franklin Lakes, NJ). Liver biopsies were collected by trans-thoracic technique, using a 16 gauge biopsy needle (Jorgensen Laboratories, Loveland, CO) (Pearce et al., 1997). All copper determinations were made through the use of atomic absorption spectrophotometry (Perkin-Elmer 2380, Norwal, CT) (wavelength, 324.7 nm) with a previously described method (Tessman et al., 2001).

The apparent prevalence (AP) was calculated by dividing the number of positive tests, those with a serum copper concentration less than or equal to 0.45 μg/g, by the total number of calves sampled at each time period.

Samples of sets of 10 and 15 serum copper results were randomly drawn without replacement from the 33 serum copper determinations. For samples of each size (n = 10 or 15), 1000 random sampling iterations were performed using a computer software program (S-Plus 2000, Mathsoft Inc., Seattle, WA). The number of positive test results, (serum copper ≤ 0.45 μg/g) was determined for each sample set. Given the number of positive tests (k) and the sample size (n = 10 or 15) the 95% lower confidence limit for prevalence was calculated for each sample. The mean and standard deviation was then

calculated for the 1000 sample sets of 10 and 15 observations (PROC MEANS, SAS Institute, Cary, NC). These results were then compared to real prevalence of copper deficiency of this population of 33 calves. Real prevalence was calculated by dividing the number of calves with liver copper concentrations less than 25 μg/g by the total number of calves. Liver copper concentrations less than 25 μg/g is the accepted test endpoint for determining copper deficiency (Puls, 1994).

For comparison purposes the estimates of the lower confidence limit calculated using the binomial model were compared with the lower 95% confidence limit calculated using the Z-distribution. Briefly, using the previously described 2 sets of 1000 randomly selected samples, the real prevalence of copper deficiency was calculated for each sample of 10 or 15 serum copper determinations using Eq 1. Thereafter, for each sample the lower 95% confidence limit of calculated real prevalence was calculated using the Z-distribution:

$$95\%\ \text{lower confidence limit} = RP - 1.96\sqrt{(RP(1-RP)/n)}.$$

Where, RP = calculated real prevalence, and n = sample size (either 10 or 15)
Thereafter, the mean and standard deviation of the 95% lower confidence limit was calculated for the 1000 randomly selected sample sets containing either 10 or 15 observations.

RESULTS

Apparent prevalence based on serum copper concentration was 0.67. Real prevalence based on liver copper concentration was 0.67. The equality of these numbers was coincidental. Results of the model verification trials are as follows. Mean and

standard deviation of the 95% lower confidence limit of prevalence calculated using the binomial model of the 10 and 15 sample trials were 0.49± 0.24 and 0.64± 0.19, respectively. Using the population proportion estimation of the 95 % lower confidence limit, the mean and standard deviation of the 10 and 15 sample trials were 0.95± 0.14 and 0.99± 0.06, respectively.

DISCUSSION

Application of this model is straightforward. If we sample 5 calves and 4 calves have serum copper concentrations less than 0.45 μg/g, we can confidently assume that herd prevalence of copper deficiency exceeds 40% (Figure 7). Three positive tests assure us that prevalence is greater than 5%. Two positive tests are consistent with a population with a 0% prevalence of copper deficiency. Cursory appraisal of these results suggests that sample sizes this small should be avoided. As sample size increases, the proportion of positive tests required to reject the hypothesis that prevalence is less than or equal to the hypothesized prevalence becomes smaller. For example, 4 of 5 tests must be positive to reject the hypothesis that prevalence is less than 40%; however, only 5 of 7 or 6 of 9 tests must be positive to reject the same hypothesis (Figure 7).

The estimates of lower confidence limits generated using the binomial model and the Z-distribution differed substantially (Figure 8). The Z-distribution produced narrow confidence limits; however, these confidence limits are clearly flawed. Lower limits calculated using the Z-distribution exceeds real prevalence for both sets analyzed, whether they contain 10 or 15 observations. In contrast, lower confidence limits calculated using the binomial model were less than the real prevalence, and hence, are

more plausible and accurate. Comparison of the results of the two methods indicates that use of the traditional method to calculate real prevalence, when the test for evaluation is substantially imperfect, is ill advised. These results re-enforce the need for novel approaches with which to interpret imperfect test results. The binomial model suggested here provides a satisfactory method with which to interpret imperfect test results.

These results highlight some interesting relationships between test results and individual test sensitivities and specificities. In particular they illustrate the potential for erroneous conclusions when results of imperfect tests are taken at face value. A large proportion of the calves are copper deficient based upon apparent prevalence. If we were to assume a herd had a real prevalence of copper deficiency of 100% we could use Eq 2 to calculate the extreme value of apparent prevalence one could expect. In this instance, AP = 1*0.53 + 1*0.89 – 1 – 0.89 +1= 0.53. This illustrates that apparent prevalence results based on serum copper concentrations over 53% predict a real prevalence over 100%.

In summary, the described procedure provides a de facto hypothesis test for prevalence. When the calculated probability is less than 0.025, we have established that the probability of the observed pattern of test results at the hypothesized population prevalence is less than 2.5%. In essence, we reject the null hypothesis that population prevalence is less than the hypothesized prevalence. In this manner we are constructing a lower limit confidence interval for herd prevalence.

The described technique has merit beyond the topic of copper deficiency. Rather than discount imperfect tests as invalid, we envision using this procedure to develop confidence limits for population prevalence in those instances in which test performance,

either sensitivity or specificity, of diagnostic tests is sub-optimal. These confidence limits will be appropriate for use in the development of disease control strategies.

CHAPTER 5

IMMUNOLOGIC EFFECTS OF COPPER SUPPLEMENTATION ON MILK FED CALVES

INTRODUCTION

Copper is an essential component of several enzymes, required to maintain host homeostasis. Included are superoxide dismutase, cytochrome oxidase, lysol oxidase, ascorbic acid oxidase and ceruloplasmin (Larson et al., 1995; Smart et al., 1992). Additionally, copper is required for iron metabolism and the prevention of cellular oxidative damage. Copper has a wide range of activities relative to the host immune response. Ceruloplasmin, a copper containing enzyme, is an acute phase protein. During inflammation, hepatic ceruloplasmin production increases dramatically in order to scavenge oxygen radicals released by immune cells (Percival, 1998). Copper-zinc superoxide dismutase (SOD) functions to eliminate oxygen radicals by reducing them to oxygen and hydrogen peroxide. The copper containing portion of this molecule functions as the superoxide-binding site. Studies have shown that SOD is critical to antioxidant defense (Percival, 1998).

Although several functions of the immune system require copper, less is known about the mechanisms and role of copper in these immune responses. Copper deficiency reduces the effectiveness of the acquired immune response. Interleukin-1 production is increased, and IL-2 production is decreased. Calves born to dams fed copper deficient diets have increased levels of tumor necrosis factor (Gengelbach and Spears, 1998). Proliferation of lymphocytes in response to mitogen stimulation in copper deficient mice

is decreased, (Kelley et al., 1995) however copper deficiency has little effect on lymphocyte blastogenesis in cattle Ward et al., 1997).

In humans and mice neutropenia is a hallmark of copper deficient states (Hayton et al., 1995; Tamura et al., 1994; Wasa et al., 1994). Bone marrow aspirates from copper deficient individuals show maturation arrest. Anti-neutrophil antibodies have been detected in copper deficient patients. Furthermore, neutrophils from copper deficient people have impaired function, including decrease in superoxide anion production and decreased candidacidal activity with normal phagocytosis. Expression of the adhesion molecule CD11b is reduced on neutrophils from copper deficient subjects (Kelly et al., 1995). Neutrophilia and increases of fibrinogen concentration have been observed in copper deficient calves. Phagocytosis of *Staphylococcus aureus* was not affected, but bacteriocidal activity may have been decreased (Arthington et al., 1996). A recent study in 6 female cattle fed a copper deficient diet showed a significant decrease in B-lymphocytes and reduced neutrophil activity. The authors interpreted this as a possible contributor to a greater incidence of infectious diseases in copper deficient cattle (Arthington et al., 1996). In addition, Gengelbach and Spears found that calves fed a molybdenum supplemented diet in order to induce copper deficiency had decreased SOD activity and serum antibody response to porcine erythrocytes (Gengelbach and Spears, 1998). Calves grazing endophyte-infected fescue had low serum copper and ceruloplasmin concentrations. A decreased monocyte phagocytic activity and expression of major histocompatibility class II antigens was observed (Saker et al., 1998). Although it is unclear from this study whether the compromised immune function is due to the

copper deficiency or to the endophyte intoxication, this model resembles husbandry in a large portion of the United States.

There is a well accepted association between copper deficiency and acute respiratory distress syndrome (ARDS) in human patients (Ho et al., 1998). This seems contrary to the pathophysiological consequences of copper deficiency. Copper deficiency is known to adversely effect neutrophil development and function (Percival, 1998). With the knowledge that a large portion of lung injury in ARDS is due to the influx of neutrophils, (Jernigan et al., 2004) one would conclude that copper deficiency should have a protective effect in these patients.

Recently Lentsch et al, believe they have found an explanation for the clinical observation of increased lung injury with copper deficiency. In a model of acute lung injury in rats, investigators found a significant increase of matrix metalloproteinase (MMP) specifically MMP-2 and MMP-9 in copper deficient versus copper replete mice. Enhanced lung injury in this model was attributed to this augmented inflammatory cell independent MMP production. The augmented MMP levels identified were unrelated to alveolar macrophage or neutrophil cell populations. The investigators concluded that the increased MMPs were a result of lung epithelial and/or endothelial cell stimulation (Lentsch et al., 2001).

The goal of this study was to define the effects of copper supplementation on immune function in calves maintained on a copper restricted diet. Selected assays of innate and acquired immune responses were performed to evaluate the effects of copper supplementation. We chose to evaluate neutrophil cytochrome c reduction and serum antibody responses to vaccination with a modified live vaccine for the common bovine

viral respiratory pathogens IBR, BVD, PI_3 and BRSV. In addition, bronchoalveolar lavage was used to determine the effects of mild neutrophil recruitment in copper deficient and replete animals on lavage fluid gelatinase expression.

MATERIALS AND METHODS

Experimental Timeline

Eleven Holstein bull calves less than one week of age were fed a whole milk diet for 26 weeks, in an effort to induce copper deficiency (Naveh et al., 1981). All calves were assessed for failure of passive transfer by serum total protein within 48 hours of age. Liver copper determinations were made at weeks 12, 24 and 26. Liver copper concentrations from week 12 were used to assign calves to two matched experimental groups based on copper status. The group to receive copper supplementation was chosen at random (Table 5). One group of the calves received copper supplementation via sustained release copper bolus during week 16 and the other did not. Calves received a modified live respiratory vaccine, administered according to label directions (intramuscularly), containing IBR, BRSV, BVD and PI_3 during weeks 16 and 20 (Bovi-Shield ® Gold 5, Pfizer Animal Health, Exton, PA). Serum samples to determine antibody titers to IBR, BRSV, BVD, and PI_3 were collected during weeks 16, 24, and 26. Bronch-alveolar lavages were performed at weeks 24 and 26. At that time samples were taken to assess lung neutrophils, lavage fluid MMP production and peripheral blood neutrophils cytochrome c reductase activity.

Liver Copper Analysis

Liver copper concentration analysis was performed similar to previously described methods (Osheim, 1983; Osheim and Ross, 1985). Liver samples were analyzed by atomic absorption using a Perkin-Elmer 2380 AAS at 324.7 nm. Liver samples were placed in a tared Teflon vials and sample wet weight was recorded. Concentrated HNO_3 (Nitric Acid TraceMetal Grade, 68 – 71%, Fischer Scientific Company, Fair Lawn, NJ.) was added to the vial. The sample was digested for 12 hours in an oven at 95°C. Samples were allowed to cool at room temperature and then transferred to 10 ml Falcon tubes (Falcon tube 15 ml conical, Fischer Scientific Company, Fair Lawn, NJ.). The vials were washed with deionized distilled water and this was added to each 10 ml Falcon tube. The sample solution was then diluted to a final volume of 2 ml. Copper standards of 0.1, 0.25, 0.5, 1.0, and 2.0 ppm were prepared in identical fashion. Samples were analyzed by group, with separate standard curve determinations for each group. Standard curve equations were produced by regression analysis. Final liver copper concentration calculations resulted from comparison to group standard curves and normalized to starting wet weight.

Serology for antibodies recognizing IBR, BVD, PI_3 and BRSV

Antibody titers of the common bovine viral respiratory pathogens IBR, BVD, PI_3 and BRSV were determined in order to evaluate humoral immune responses. Calves received two modified live vaccinations for these pathogens over the course of the study. The first vaccine was administered at 4 months of age. The first of the serologic evaluations was performed on serum samples from blood drawn immediately before

vaccination. Four weeks post initial vaccination calves received another vaccination in order to boost their immunologic response. At 4 and 6 weeks after this second vaccination, serum was obtained for an additional evaluation of antibody response to vaccination. Serologic assays were performed by the University of Missouri-Columbia Veterinary Medical Diagnostic Lab using a serum virus neutralization assay.

Neutrophil recruitment model

Mild lung inflammation similar to that seen in sub-clinical respiratory disease was induced by saline lavage of the airway (Holtzclaw et al., 2004). This model consisted of two BAL's performed two weeks apart. The first lavage induced the inflammatory response as well as provided baseline data for the two experimental groups. The subsequent lavage served to evaluate the inflammatory response. All calves were monitored closely for clinical evidence of respiratory disease.

BAL and alveolar macrophage isolation

The calves were weighed and sedated with xylazine (0.05 mg/kg IM) and placed in left lateral recumbency. A sterile BIVONA, bronchoalveolar lavage catheter was passed via the nares into a lobar bronchus (right diaphragmatic lobe). Forty milliliter aliquots of sterile phosphate buffered saline (pH 7.4, 300 mOsm) were instilled by gravity flow, until flow slowed, at which time recovery of the fluid was performed by aspiration. Aspiration of fluid was continued until resistance was encountered, at which time additional aliquots of saline were instilled. This process was repeated for 4 cycles, equivalent to instillation of a total of 160 ml of saline lavage solution. Recovered lavage fluids were immediately placed on ice where they remained until analyzed (within one

hour of collection). Cell numbers, viability, differential cytology, were performed at each time point.

Broncho-alveolar lavage cells were centrifuged in phosphate buffered saline at 400 x g for 15 minutes. The cell pellet was washed twice by centrifugation of the BAL followed by resuspension in phosphate buffered saline and centrifuged at 400 x g for 15 minutes. The BAL cell pellet was re-suspended in RPMI-1640 medium, pH 7.4 and diluted to approximately 1 x 10^6/ml. Mucous and non-cellular debris was removed by suction. Cell viability and numbers were determined using a hemocytometer and 1% Trypan blue

Differential cell counting of BALF cells were performed on Wright stained smears. One-hundred microliter aliquots were placed into a cytocentrifuge apparatus and centrifuged at 185 x g onto glass slides. Differential counts were based on counting 500 cells. Cells were classified as monocytes, lymphocytes, polymorphonuclear, eosinophil, basophil/mast cells, and epithelial cells.

Isolation of peripheral bovine neutrophils

A method similar to that described by Carlson and Kaneko was used to isolate neutrophils from peripheral blood for use in cytochrome c reduction determination. Briefly, four 10 ml vacutainer tubes with ACD coagulant were used to obtain peripheral blood, (ratio of blood to ACD 9:1). All samples were kept on ice for transport to the laboratory. The blood was centrifuged in a 50 ml conical vial at 1000 x g for 10 minutes at 4 C with the brake off. Plasma, the buffy coat layer and top quarter of red blood cells (RBC) layer were removed until a final volume of 10 ml of RBC pellet remained. The

cell pellet was resuspended with 10ml of HBSS. The remaining RBCs were lysed by addition of 25 ml of endotoxin free ACK lysis buffer and incubation in a water bath at 37 C for 10 minutes Tubes were then be centrifuged at 500 x g and 4 C for 8 minutes with the brake on. The resulting pellet was resuspended with 40 ml of HBSS. The wash procedure was repeated twice. Trypan blue exclusion was used to determine cell viability. One-hundred microliter aliquots were placed into a cytocentrifuge apparatus and centrifuged at 185 x g onto glass slides. Purity of neutrophil preparation was determined by counting at least 200 cells/slide on Wright stained preparations. Only preparations with > 90% neutrophils with > 90% viability were utilized in the study.

Cytochrome c reduction activity

Stimulant Preparation – Phorbol myristate acetate (PMA) was dissolved in 1 ml of DMSO to a final concentration of 1 mg/ml and stored at –20˚C. Phorbol ester stock solutions were diluted to 1:100 (10 ng/µl) for use in the assay.

Determination of PMN Superoxide Metabolism – Two million (2 x 10^6) cells were stimulated with three PMA concentrations (PMA 30, 60, and 300 ng/ml). Superoxide production was performed as described (Kuthan et al., 1986). Final concentrations of cytochrome C was 50 uM. Twenty units of superoxide dismutase (SOD) were added to each of the PMA controls. After 20 minutes at 37˚C, the supernatant was decanted into disposable cuvettes and cytochrome C reduction was determined by scanning between 530 – 570 nm. Cytochrome C reduction was determined by subtracting the absorbance at 550 nm from baseline (530 nm) and multiplying by the molar extinction coefficient of horse heart cytochrome C. Each value was normalized to total cell count (nmole reduced/2 x 10^6 cells).

Western blot for metalloproteinase identification in BAL fluid

BAL fluid (35 µl) was separated in a denaturing 10% polyacrylamide gel and transferred to a 0.1-µm-pore nitrocellulose membrane. Nonspecific binding sites were blocked with Tris-buffered saline (TBS; 40 mM Tris, pH 7.6, and 300 mM NaCl) containing 5% nonfat dry milk for 2 h at room temperature. Membranes were then incubated overnight at 4°C in 0.5 µg/ml of polyclonal sheep anti-MMP-2 or anti-MMP-9 (Biogenesis sheep anti-human MMP 2 or MMP 9;) in TBS with 0.1% Tween 20 (TBS-T). After three washes in TBS-T, the membranes were incubated for 2 h in 0.15 µg/ml of horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) at room temperature. Membranes were washed three times in TBS-T, and immunoreactive proteins were detected by enhanced chemiluminescence. Positive controls from affinity purified bovine MMP 2 and MMP 9 were run in adjacent wells for each gel.

Gelatin zymography for evaluation of metalloproteinase activity in BAL fluid and alveolar macrophages

SDS-PAGE gels (10%) containing 1mg/ml of gelatin were prepared. Denatured but nonreduced BAL fluid samples were electrophoresed into the gels at constant voltage. The SDS was removed from gels by washing twice (20 min/wash) in water containing 2.5% Triton X-100 at room temperature and incubated overnight in gel development buffer (10 mM Tris ·HCl, pH 7.5, 1.25% Triton X-100, 5 mM $CaCl_2$, and 1 µM $ZnCl_2$) at 37°C. Gels were stained with Coomassie blue for 3 h and then destained. Regions of negative staining indicated the presence of proteinases with gelatinolytic activity. For

each gel, a standard containing recombinant human fibroblast MMP 9 and bovine peripheral blood PMN MMP 9 were loaded into one of the wells for comparison of the molecular weights of gelatinolytic bands present in the BAL fluid (Figure 9). Bovine MMP 9 (multimer, pro-MMP 9, active MMP 9) was activated using trypsin activated recombinant human stromelysin I. In addition, 2 mM amino-phenyl mercuric acetate was used to chemically active MMP 9 for comparison with enzymatically activated enzyme. These two controls were necessary to decipher the pattern present in calf BAL fluid. The relative gelatinolysis was determined using Scion Image for Windows (www.scioncorp.com) on an inverted image (gelatinolytic bands dark on a white background). Dark band (0-255) area was determined and raw area units (dimensionless) were used as a qualitative determination of activity (relative to standards).

Statistical analysis

All considered dependent variables were analyzed using either a two or three factor repeated measures ANOVA with the assistance of a statistical software package (PROC GLM, SAS institute, Gary, Indiana). Any significant differences were evaluated using the least squares difference method. Dependent variables analyzed included liver copper concentration, serum antibody titers to IBR, BVD, BRSV and PI_3, systemic neutrophil cytochrome c reduction, and BAL lymphocyte, neutrophil, macrophage percentages and neutrophil to macrophage ratio. Independent variables considered included subject, treatment (copper supplementation), time, and time and treatment interactions. Analysis of neutrophil cytochrome c reduction included level of PMA stimulation as an independent variable. Pro-MMP 9, Pro-MMP 2, active MMP 9, active MMP 2, active percent of both

MMP 9 and MMP 2 and total MMP production were analyzed with a two factor repeated measures ANOVA. In all analyses a p-value of less than or equal to 0.05 was considered significant.

RESULTS

All calves had adequate passive transfers as their serum total proteins were above 5.0 mg/dl. Only 3 of 11 calves (27%) were copper deficient based on a liver copper concentration of less than 25 μg/g prior to copper supplementation. The rest of the calves had liver copper concentrations greater than 25 μg/g. Liver copper concentrations ranged from 21 to 73, had a mean of 40 μg/g and a standard deviation of 18 μg/g. Week 24 liver copper determination, 8 weeks post supplementation, indicated that 1 of 5 control calves were copper deficient and all treated calves were copper replete. Liver copper concentrations ranged from 16 to 281 μg/g, had a mean of 126 μg/g and a standard deviation of 90 μg/g. Two factor repeated measures analysis revealed significant differences in treatment, time, and time X treatment interactions ($p = 0.001$, $p = 0.013$, $p = 0.039$ respectively). The liver copper concentrations increased with respect to treatment and time. There were no significant differences detected in serum copper concentrations (Figure 10).

In the analysis of respiratory pathogen serum antibody titers, no significant differences were found in BRSV, IBR, BVD or PI_3 serum antibody titers (Figures 11 and 12). Treatment was not a significant factor in any of the BAL dependent variable analyses. Time was a significant factor in lymphocyte percentage ($p = 0.011$). The lymphocyte percentage decreased with respect to time. There was no statistically significant change in macrophage percentage, neutrophil percentage or neutrophil to macrophage ratio.

Circulating cells isolated to evaluate cytochrome c reduction were > 90% neutrophils and > 90% viable. Although there was no significant differences in treatment main effects detected there were significant time effects, level effects, and time x level interactions ($p = 0.005$, $p = 0.001$, $p = 0.004$, respectively) with respect to cytochrome c reduction. The level of cytochrome c decreased with respect to time and increased in samples stimulated with 300 ng/ml of PMA. There were no statistically significant differences identified in the analysis of the MMP dependent variables.

DISCUSSION

The choice of Holstein bull calves as experimental subjects was one of convenience and economics. These calves are readily available and less expensive than beef calves. The feeding protocol was one that was designed to induce copper deficiency while simulating beef cattle production. Whole cows milk is low in copper relative to other species (Naveh et al., 1981). Unfortunately only in a small portion, 27%, of calves, was copper deficiency induced as measured by liver copper concentration determinations. There may be several explanations for this. All calves started with adequate copper stores as they came from well fed, productive dams. The whole milk was obtained as dump milk from a local dairy. This milk may have been higher in copper content than would be found in a beef animal on a marginal or deficient diet.

The use of copper boluses for supplementation worked well. The supplemented calves had significant increases in liver copper concentration with respect to the unsupplemented calves. This is similar to what other investigators have found (Gay and

Pritchett, 1988). Using this method relieved us from having to account with the variation that is inherent in free choice supplementation. This supplementation strategy is easy to perform and cost effective. Liver copper concentration did increase over time in all calves. Although one calf remained copper deficient this is an indication that our feeding strategy was less than optimal for inducing copper deficiency.

We saw no differences in serum antibody titers. This is similar to other investigators results (Gengelbach and Spears, 1998). Titers did not increase after a second vaccination with a modified live virus vaccine. We expected to see an elevation simply due to vaccination in all calves due to an amnestic response. This may indicate that the vaccine used poorly stimulates the immune system or that the timing of sampling did not coincide with the expected rise in antibody titers. Additionally the method used to detect antibody titers, serum virus neutralization, may not detect antibodies produced in response to this vaccine. The absence of differences due to treatment was expected. Although lymphocyte blastogenesis and antibody production are decreased in association with copper deficiency in other species, (Percival, 1999) other investigators report no effect in cattle with copper deficiency (Arthington et al., 1996). In addition our study did not measure the effect of copper deficiency directly, rather the effect that copper supplementation may have.

The only statistical difference noted in BAL cellular analysis was due to time. Lymphocyte percentages decreased, but there were no statistically significant changes in macrophage number although they did tend to decrease. These changes are consistent with evidence of general lung inflammation. This evidence is further support that this model for studying mild acute lung inflammation is valid. The fact that no significant differences attributable to treatment were found is not surprising. In other models of acute lung injury

no differences between copper deficient and copper supplemented animals were detected (Lentsch et al., 2001). Based on observations made in copper deficient rat's lung inflammation as measured by myeloperoxidase content was not different from their copper replete counterparts (Lentsch et al., 2001). We did expect to see a difference in MMP production due both to time and treatment. Other models of lung inflammation have shown that active MMP 9 is elevated in response to acute lung inflammation. Experimental models in rats indicate a dampening of this effect in copper replete subjects as compared to their copper deficient counter parts (Lentsch et al., 2001). Although the model of lung inflammation appeared to have a mild effect on BAL cell distributions, it may be that the insult was too mild to show the increases in MMP production that have been seen in previous studies. If that is the case a difference due to treatment would be very difficult to demonstrate. Copper supplementation in calves with adequate liver copper stores does not appear to have an effect on the calf's immune system. This model potentially does not adequately show the potential beneficial effects that copper supplementation may have on copper deficient calves.

Table 1. Sensitivity and specificity of serum copper determinations for detection of low liver copper concentration (25 ug/g wet weight).

	Test results					
Serum copper (ug/g)	**True positive**	**False positive**	**False negative**	**True negative**	**Sensitivity**	**Specificity**
≤ 0.40	11	7	21	66	0.34	0.90
≤ 0.45	17	8	15	65	0.53	0.89
≤ 0.50	17	14	15	59	0.53	0.81
≤ 0.55	20	24	12	49	0.62	0.67
≤ 0.60	23	31	9	42	0.71	0.58
≤ 0.65	25	38	7	35	0.78	0.48
≤ 0.70	27	40	5	33	0.84	0.45
≤ 0.75	28	44	4	29	0.87	0.40
≤ 0.80	29	48	3	25	0.90	0.34
≤ 0.85	31	55	1	18	0.96	0.25
≤ 0.90	31	57	1	16	0.96	0.22
≤ 0.95	31	66	1	7	0.97	0.10
≤ 1.00	32	68	0	5	1.0	0.07
≤ 1.50	32	73	0	0	1.0	0.0

Table 2. Apparent and calculated real prevalence of copper deficiency by agricultural region. Proportion of copper-deficient calves differed significantly by region, ($P < 0.05$).

	Calves at risk[d]	n	Deficient Calves (≤ 0.45 μg/g)	Apparent prevalence	Real Prevalence*	Number of Calves Copper Deficient
1 Northwest	223,000	86	15	0.174	0.153	34,203
2 North Central	222,000	48	9	0.188	0.185	40,964
3 Northeast	121,000	99	22	0.222	0.267	32,330
4 West Central	240,000	36	7	0.194	0.201	48,253
5 Central	460,000	81	18	0.222	0.267	122,910
6 East Central	136,000	55	16	0.291	0.431	58,580
7 Southwest	312,000	50	2	0.040	0.000 **	0
8 South Central	320,000	58	2	0.034	0.000 **	0
9 Southeast	39,000	15	5	0.333	0.531	20,738
Summary estimates	2,073,000	528	96	0.182	0.171	357,980

* Real prevalence was calculated using the formula, $RP = (AP + S_p - 1) / (S_e + S_p - 1)$, and the sensitivity and specificity, 0.53 and 0.89 respectively.

** calculated value is below zero

Table 3. Results of forward stepping regression model predicting serum copper concentration (μg/g) as a function of various husbandry practices.

Variable	Coefficient	P-value
Intercept	0.53997	<0.0001
West Central	-0.10937	0.0068
East Central	-0.10168	0.0015
Southwest	0.12965	0.0001
South Central	0.09887	0.0020
Thin cows	-0.21109	<0.0001
Calves aged 7 to10 months	0.07255	0.0019
White clover	-0.09013	0.0196
Lespedeza	0.14705	<0.0001
Creep feed	0.11264	<0.0001
Trace mineralized salt	0.04573	0.0382

Table 4. Results of logistic regression for predicting copper deficiency (≤ 0.45 μg/g) in 528 spring-born Missouri feeder calves as a function of various husbandry practices.

Parameter	Coefficient	P-value	Odds Ratio
Intercept	-0.4945	0.0204	
Southwest	-1.9913	0.0069	0.137
South Central	-2.2241	0.0029	0.108
ladino clover	0.6524	0.0225	1.920
alfalfa	-2.4606	0.0215	0.085
creep feed	-1.0001	0.0004	0.368
trace mineralized salt	-0.8770	0.0004	0.416

Table 5. Experimental group allocation for supplementation with a sustained release copper bolus.

Supplemented		Control	
Calf Number	Liver [Cu] µg/g	Calf Number	Liver [Cu] µg/g
4	24	13	21
7	28	6	34
10	25	12	43
3	40	5	45
2	46	9	73
11	71		

Figure 1. Definition of true and false positive and negative results of analysis of serum copper concentration for estimation of copper deficiency.

	True copper status	
	Copper deficient*	**Copper replete[H]**
Interpretation of serum copper analysis		
Copper deficient[‡]	True positive	False positive
Copper replete[§]	False negative	True negative

*Liver copper concentration, < 25.0 μg/g. [H]Liver copper concentration, ≥ 25.0 μg/g.

[‡]Serum copper concentration, ≤ 0.45 μg/g. [§]Serum copper concentration, > 0.45 μg/g.

Figure 2. Serum and liver copper concentrations (individual calf data points [•]) in 105 beef calves. The straight solid line represents the derived linear regression equation: Liver copper concentration = 6.384 + (109.126 X serum copper concentration); $P < 0.001$; $r^2 = 0.16$. The curved dashed line represents the derived polynomial regression equation: Liver copper concentration = -84.517 + (524.253 X [serum copper concentration]) – (560.522 X [serum copper concentration]^2) + (228.374 x [serum copper concentration]^3); $P = 0.01$; $r^2 = 0.17$.

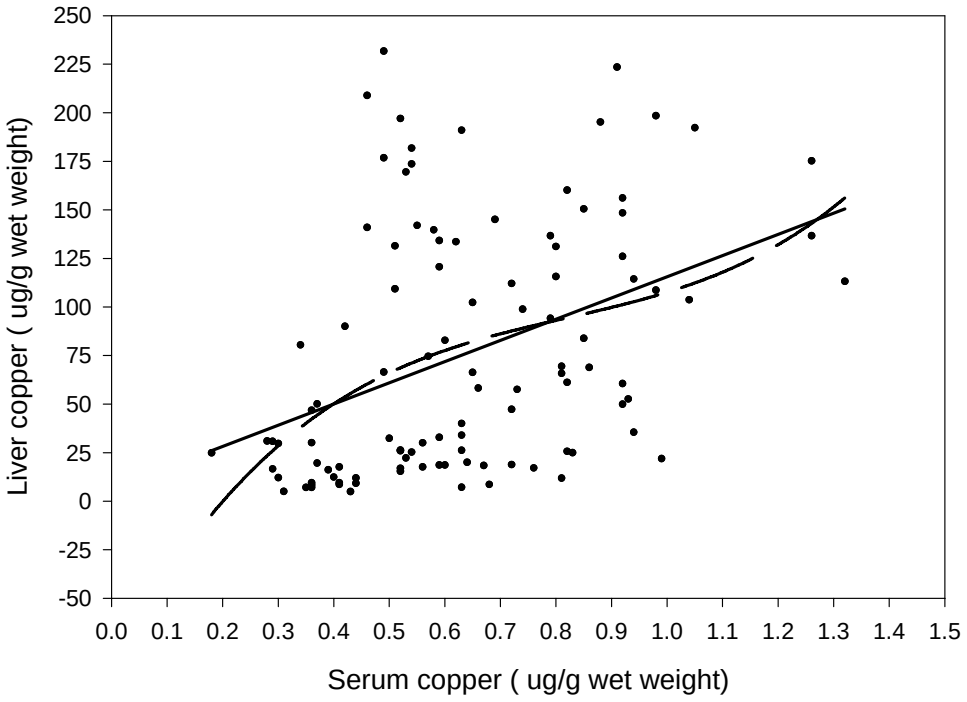

Figure 3. Real prevalences calculated for all possible apparent prevalences (11 to 53%), using serum copper concentration test endpoint of ≤ 0.45 ug/g, sensitivity of 0.53, and specificity of 0.89.

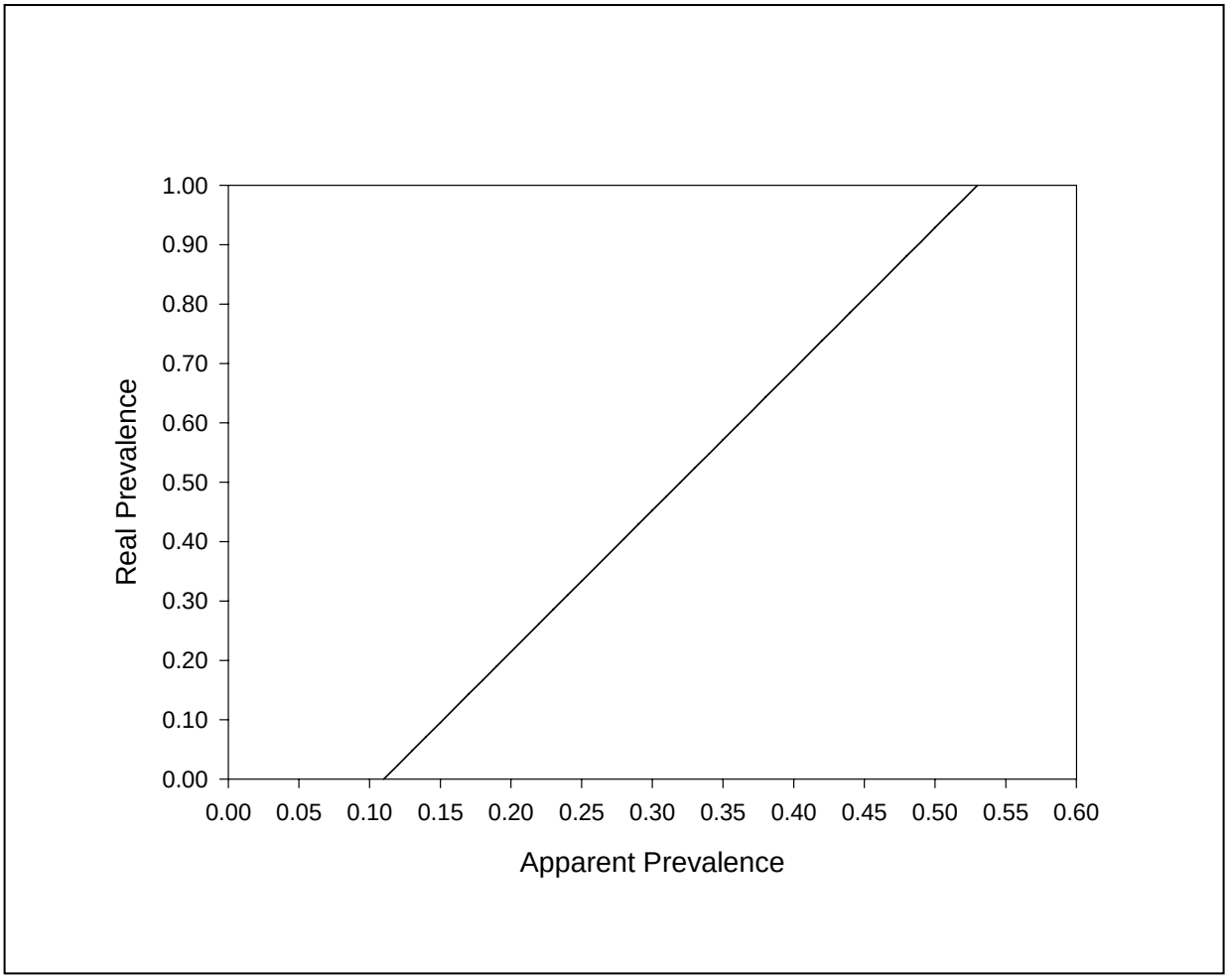

Figure 4. Predictive values of positive and negative test results calculated by use of serum copper concentration test endpoint of ≤ 0.45 ug/g and all possible prevalences of copper deficiency. Solid line indicates relationship between real prevalence and predictive value of positive test results. Dotted line indicates relationship between real prevalence and predictive value of negative test results.

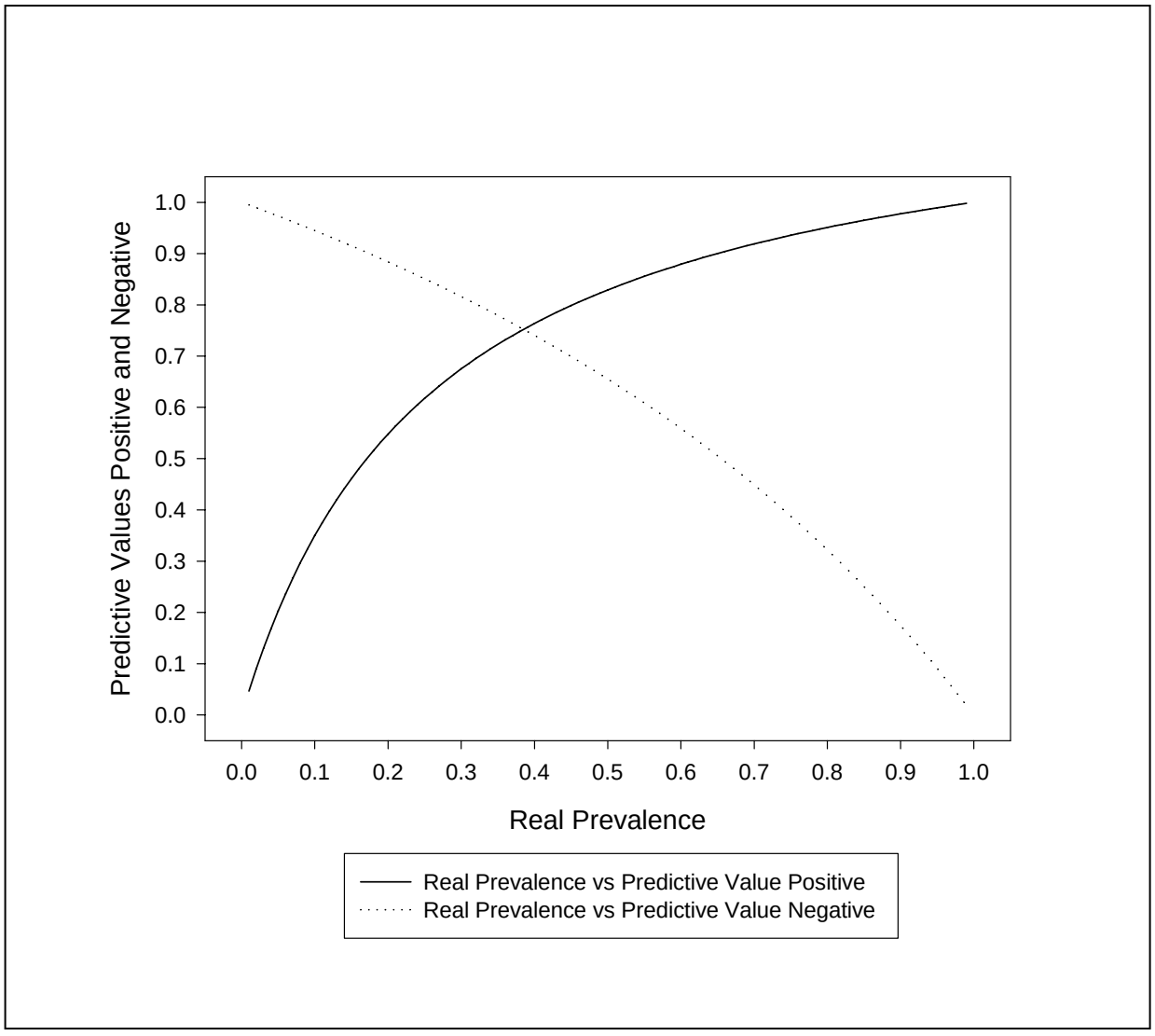

Figure 5. Proportion of calves within each of eight classes of serum copper concentration. The numbers appearing at the top of each bar represent the total number of calves in each class. The vertical dashed line (- - -) represents the test endpoint for copper deficiency (≤ 0.45 μg/g). Classes are intervals of serum copper concentration of 0.15 μg/g, except for the last class, which is any value greater than 1.05 μg/g.

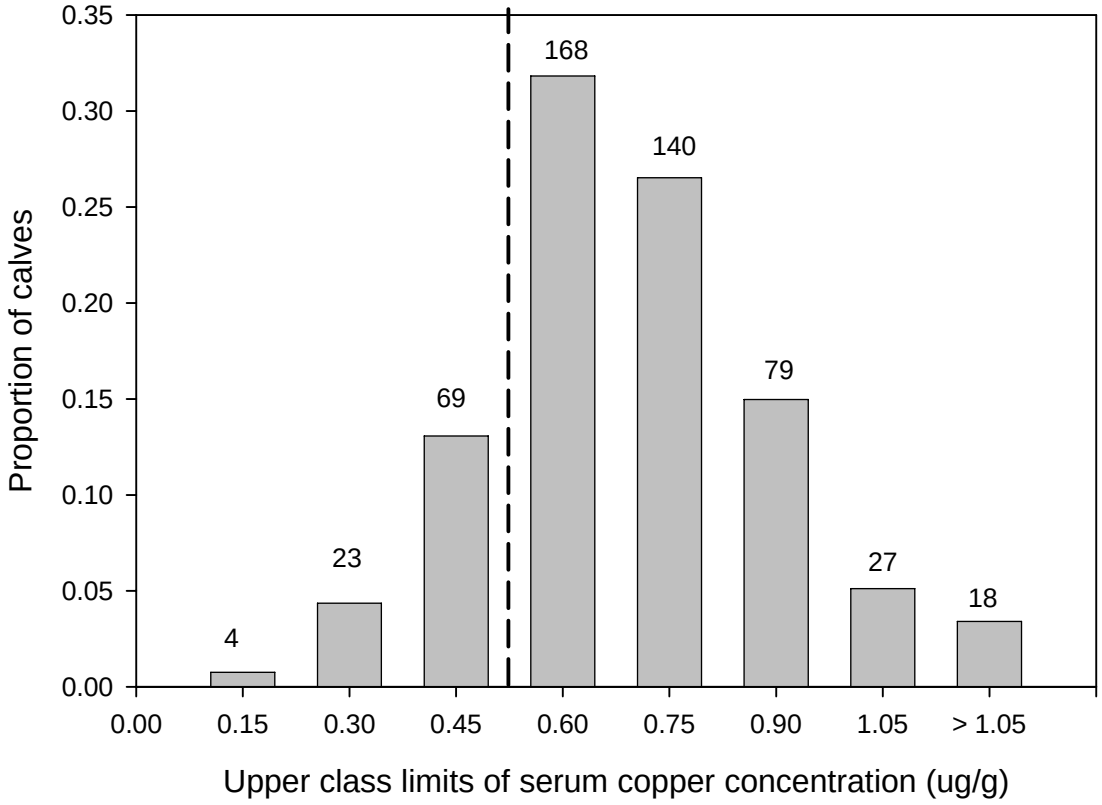

Figure 6. Map of Missouri depicting the 9 agricultural districts and the Missouri and Mississippi rivers. Gray shaded regions have estimated real prevalence of copper deficiency greater than 20%. Key for districts; 1= Northwest, 2= North Central, 3= Northeast, 4= West Central, 5= Central, 6= East Central, 7= Southwest, 8= South Central, 9= Southeast.

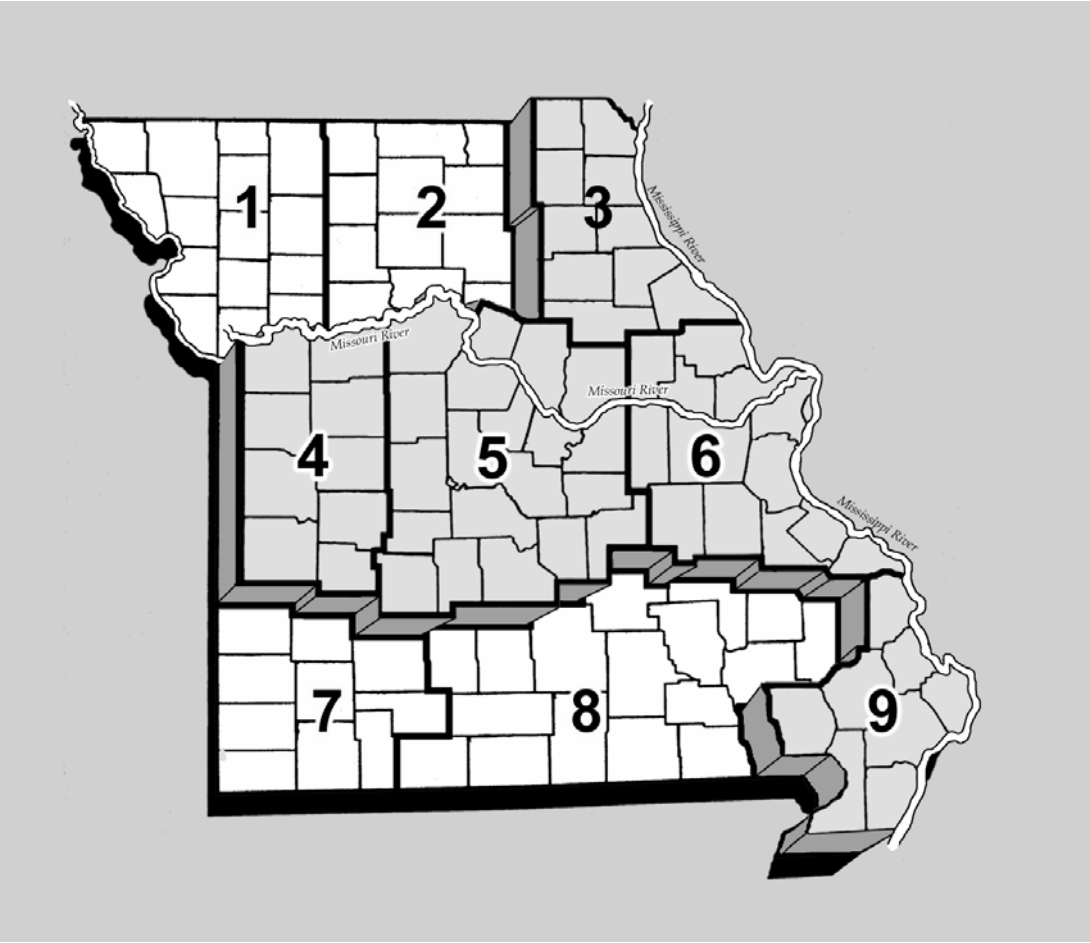

Figure 7. Interpretation of test outcomes for various combinations of number of tests and number of positive test results. Cells designated by an asterisk (*) denote the calculated lower limit of copper deficiency prevalence.

Tests performed	Positive Test results	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95
5	5																	*		
5	4								*											
5	3	*																		
6	6																			*
6	5											*								
6	4					*														
7	7																			*
7	6														*					
7	5								*											
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10	9																			*
10	8															*				
10	7											*								
10	6							*												
10	5			*																

Figure 8. Results of 2 trials of randomly generated serum copper concentration sample selection outcomes for estimation of the lower 95% confidence interval of prevalence of copper deficiency. The • represents the mean of each trial and the solid horizontal line represents the standard deviation of the proposed binomial model. The ∇ represents the mean of each trial and the solid horizontal line represents the standard deviation of the traditional method of determination. The vertical dashed line represents the real prevalence of the population from which the samples were taken as determined by liver copper concentrations.

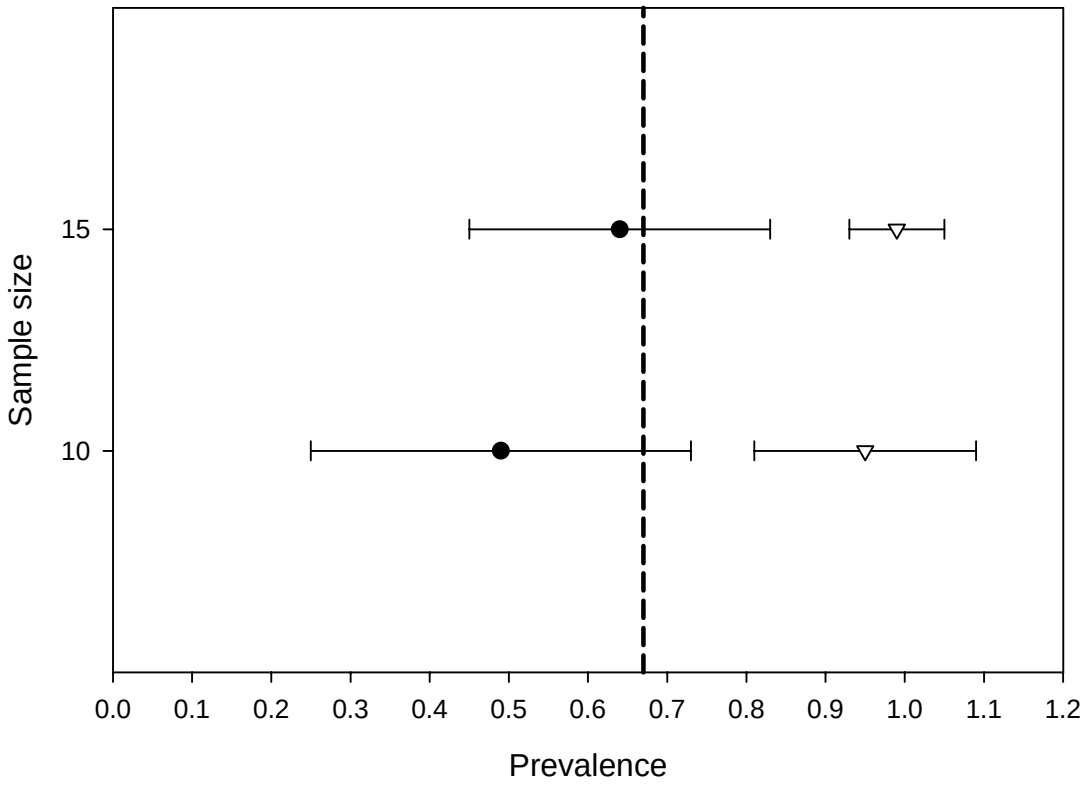


Figure 9. Photograph of zymography gel used to determine relative activities of MMP subtypes.

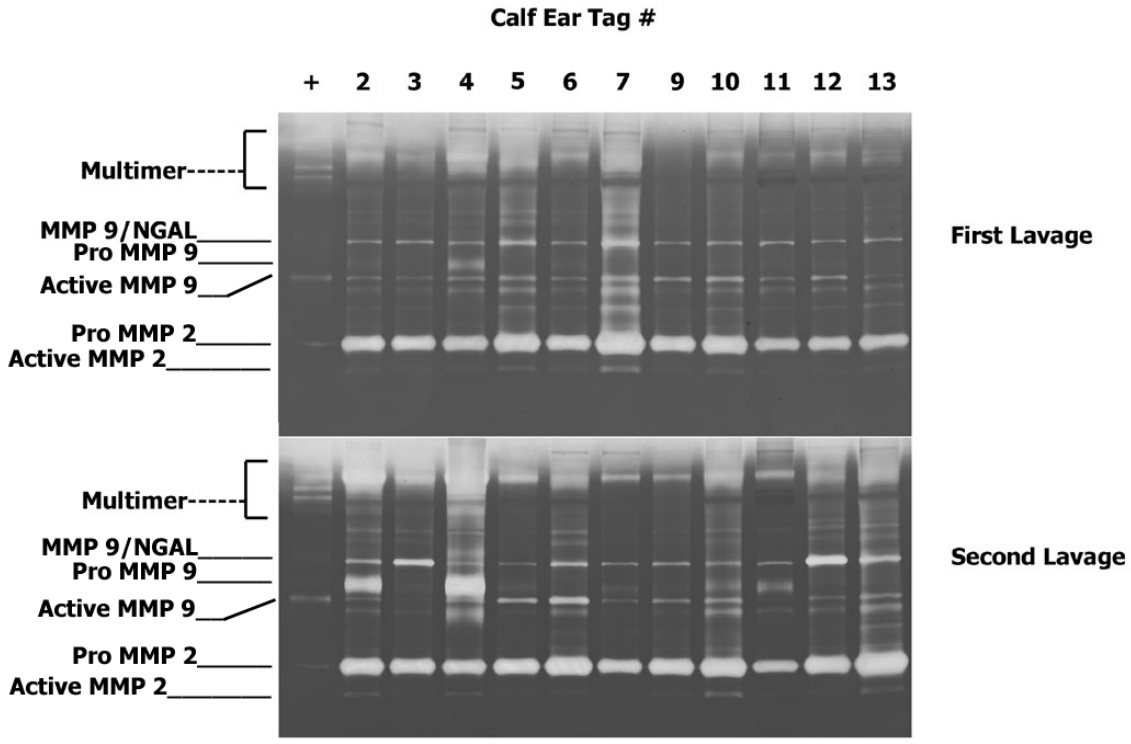


Figure 10. Comparison of liver copper concentration (µg/g) between supplemented and control groups.

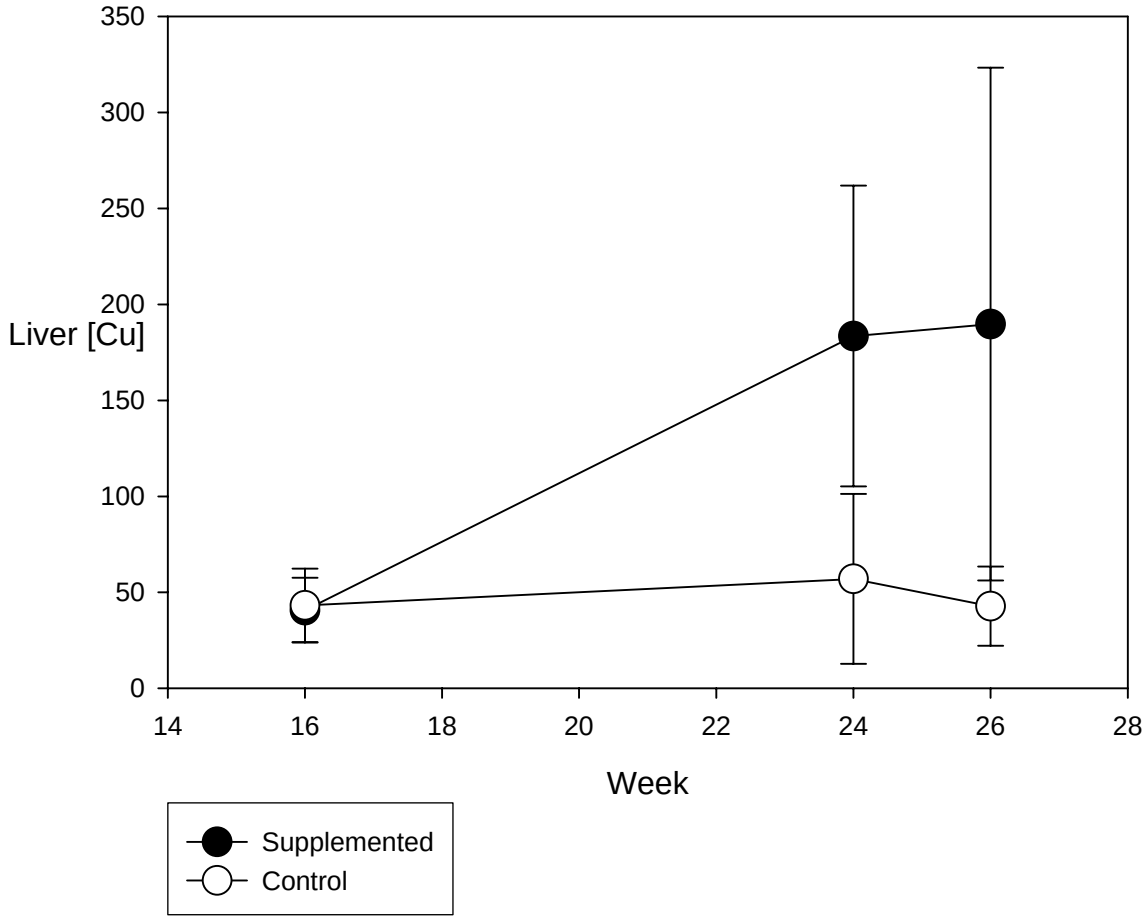


Figure 11. Comparison of serum titers for BRSV between supplemented and control groups.

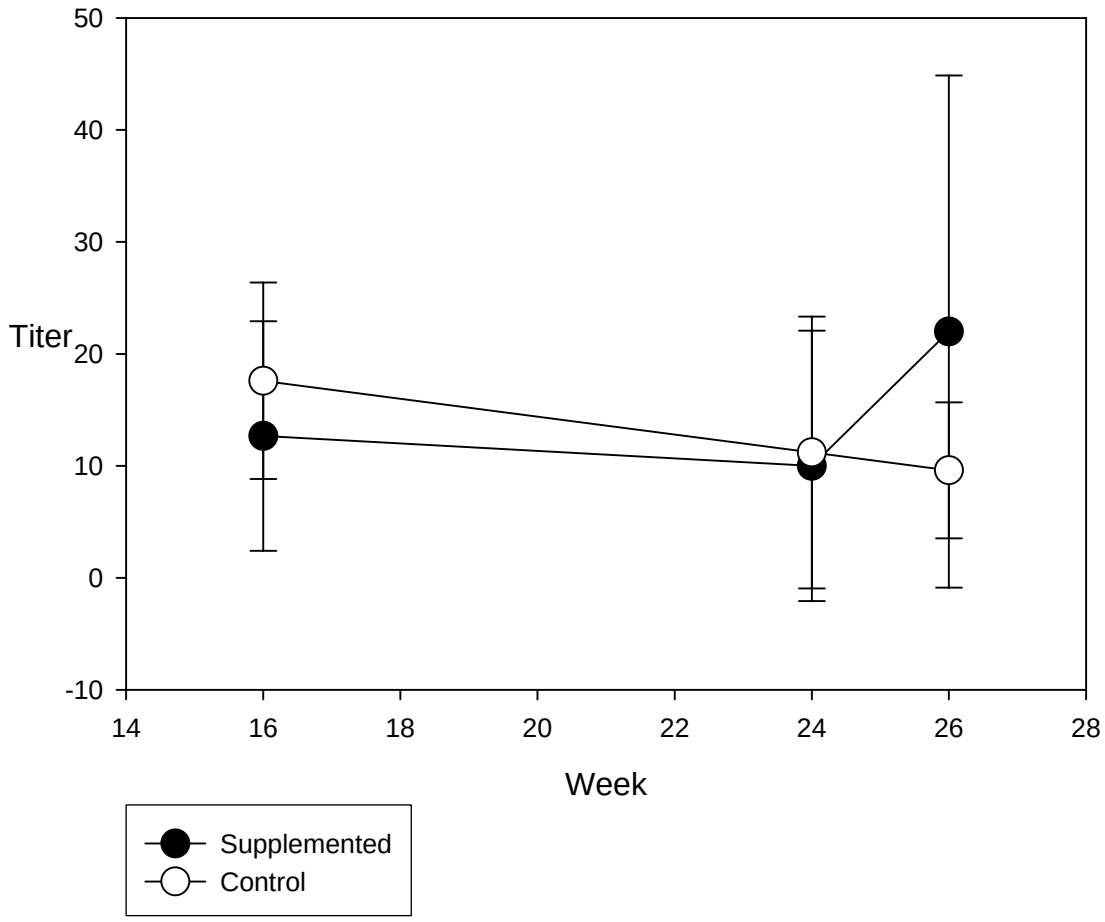
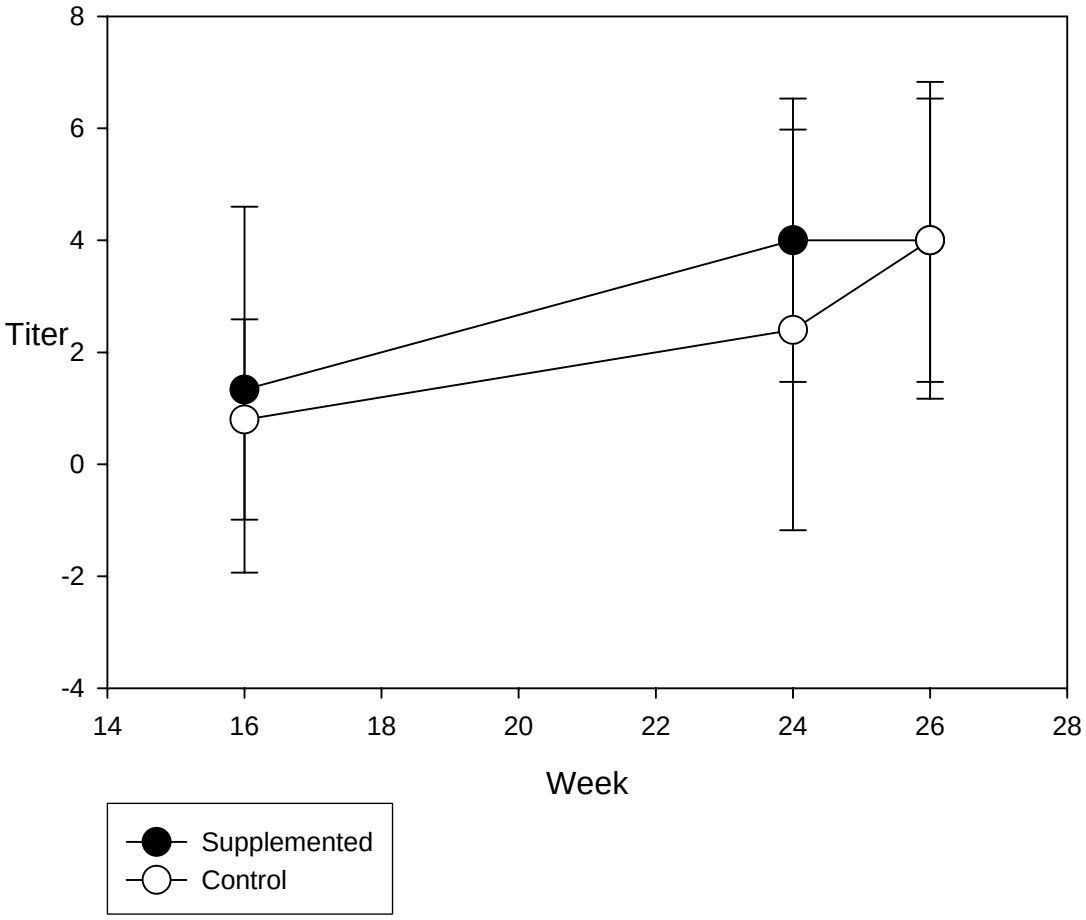


Figure 12. Comparison of serum titers for IBR between supplemented and control groups.



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VITA

Ronald Kenneth Tessman was born as baby boy Birchfield, on September 24th, 1969, to James Daniels and Alice Birchfield in Great Falls, Montana. Shortly thereafter he was adopted by Lawrence and Margaret Tessman. Due to divorce he spent a short time in foster homes and a local orphanage. After being reunited with his adopted father, Lawrence Tessman he grew up and attended public schools in Great Falls, Montana.

Ronald attended Montana State University for his undergraduate education. He enrolled in the chemical engineering program, but switched to medical microbiology when he decided that he would like to attend veterinary school. Ronald attended Washington State University for veterinary school. He graduated with a bachelor of science in 1994 and with a Doctor of Veterinary Medicine in 1997 from that institution. He spent one year at Kansas State University completing an internship in Agricultural Practices. He then attended the University of Missouri and completed a large animal internal medicine residency and a doctoral degree program. He was awarded board certification in Large Animal Internal Medicine in May 2005.

Currently Ronald is employed at the University of Missouri as clinical instructor in the Food Animal Medicine, Surgery, and Production Medicine section. He is also course coordinator and lead lecturer in the Veterinary Epidemiology and Biostatistics and Veterinary Public Health courses. He resides outside of Hallsville, Missouri, with his wife, Tanya Tessman and children. Ronald has five children, Jessica Spencer, Hunter Tessman, Brittany and Brandon Borlinghaus and Faith Tessman