

EQUINE SALMONELLOSIS: SEROLOGY AND CELL MEDIATED  
IMMUNITY IN NATURALLY INFECTED CARRIERS AFTER  
HYPERIMMUNIZATION WITH AN  
AUTOGENOUS BACTERIN

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## CHAPTER I

### INTRODUCTION

Bacteria in the genus *Salmonella* are a significant cause of morbidity and mortality in horses, especially foals which often die of an acute septicemia (1). Adults may also develop a septicemia but more commonly have an acute or chronic enteritis depending on which serotype causes the infection (2, 3). Recovered cases may become carriers and shed the bacteria in their feces for long periods during and after their convalescence. These horses contaminate the environment, acting as potential sources of infection for other horses and man, and increase the difficulty of controlling the disease (3, 4, 5). A means of identifying carriers would be very helpful. These horses could then be isolated to reduce the chance of further spread (3, 6). More knowledge about the carrier state of salmonellosis in horses, including tests to identify these animals, would be valuable to equine clinicians and owners.

Diagnosis of bacterial enteric infections is accomplished by culturing feces. Since there are numerous bacteria in such samples, enrichment techniques that encourage the growth of *salmonella* while discouraging the growth of other bacteria are required (7). Fecal culturing is time consuming and expensive and may not detect some cases. A series of consecutive negative samples from an individual horse have been followed by isolation of the bacteria (8). Diagnosis of

salmonellosis in the inapparent carrier by fecal culture is difficult unless the horse is stressed and sheds the bacteria. Even then a fecal culture could be negative if there are low numbers of the organism present in the sample (8).

Pathogenic salmonellae, Salmonella enteritidis typhimurium in particular, can survive and even multiply within cells of the reticulo-endothelial system (RES) (9, 10). Studies show that antibodies are able to reduce the severity of infection; but, cell mediated immunity (CMI) and activation of macrophages are the most significant responses for prevention or cure of salmonellosis (11, 12).

Salmonella vaccines are still used in equine medicine, although not as frequently as in the past. Autogenous bacterins, prepared from Salmonella enteritidis serotype abortusequi appeared to have significantly reduced abortions caused by infection from this serotype (a host-specific salmonella that is no longer isolated in this country). Bacterins prepared from other serotypes have had varying degrees of success. The best results have been obtained with bacterins to serotype typhimurium, used to vaccinate broodmares and improve foal survival on infected ranches (6, 13, 14, 15).

Hyperimmunization, a series of injections of a bacterin given over a short period of time, has not been studied in salmonella carrier horses. The objective of this study was to evaluate the response of naturally infected carrier horses to hyperimmunization with autogenous bacterins. The horses used in this study were infected with Salmonella enteritidis serotypes, braenderup or agona. Immunological and clinical responses, fecal culture, and postmortem examination, were the basis for the evaluation.

## CHAPTER II

### REVIEW OF THE LITERATURE

#### The Genus *Salmonella*

*Salmonella* are members of the family Enterobacteriaceae, a very large and diverse group. This family consists of gram negative, medium-sized rods, which are facultatively anaerobic, oxidase negative, non-sporeforming and fermentative bacteria. They are found in the intestines of man and animals, in the soil and on plants (16). The most recent classification scheme gives the genus three species. These are *Salmonella choleraesuis*, *Salmonella typhi* and *Salmonella enteritidis* which is further subdivided into serotypes. There are over 1700 serotypes of *Salmonella enteritidis*. Since the serotype is not a true subspecies, the nomenclature is variable, and one may see many ways of identifying the bacteria in the literature (17).

The older classification system, the Kauffman-White Scheme, established relationships of salmonella called groups, which were based on shared common O antigens. The somatic or O antigen is a heat-stable polysaccharide associated with the body of the cell. Knowledge of the polysaccharide makeup of salmonella has demonstrated the sensitivity of the Kauffman-White classification scheme (18). The most striking difference between virulent and less virulent serotypes is related to the outer portion of the cell wall which is also responsible for O

antigenicity (19). Most clinical isolates from man and animals were found in groups A through O (16).

Besides the somatic antigen, there is also a flagellar or H antigen, which is heat labile and present in all motile salmonella. There are two genes that determine the structure of the flagellar filament subunit protein, flagellin. These genes are located on opposite sides of the genome and are expressed alternatively. Switches from one phase to the other occur at frequencies of about  $10^{-3}$  to  $10^{-5}$  per cell generation (20).

Phage typing and DNA hybridization have also been used to characterize salmonella isolates. Epidemiological studies using phage typing helped determine that birds were the most likely source of an outbreak since different phage types appear to be associated with certain hosts (10). Although unavailable in diagnostic laboratories at present, DNA hybridization may be the most accurate method of identifying the bacteria. This method has been especially useful when studying independent phenotypic factors not affected by plasmid-transferred genes (21, 22).

#### The Disease in Horses

The most common serotypes of salmonella associated with disease in horses are Salmonella enteritidis serotypes typhimurium, enteritidis, anatum, heidelberg and newport (2, 3). Other serotypes have been involved in outbreaks and in individual cases. Salmonella enteritidis serotype agona was found in a number of California horses but did not appear to be especially pathogenic (3, 23). Serotype typhimurium accounts for at least 60 percent of the isolations in most countries (2).

Ingestion is the most common mode of infection for horses although the bacteria has been known to enter through mucous membranes (16).

Salmonella are facultative intracellular parasites that localize in cells of the RES and associated lymph nodes (24, 25). Invasive strains may be taken up by macrophages and spread via the lymphatics (1, 3, 10, 26). The pathogenesis of salmonellosis depends on the ability of the organism to: invade the gastrointestinal mucosa, cause diarrhea, and disseminate from the intestine and multiply within the RES (27). The most severe gastrointestinal lesions associated with equine salmonellosis are located in the distal ileum, proximal colon and cecum (28). At postmortem, the best tissues to collect for possible salmonella isolation are mesenteric lymph nodes, mucosa of the cecum and proximal large colon, distal ileum, the liver and the spleen (3).

Clinically salmonellosis in horses can be divided into categories based on time of onset of clinical signs after introduction of the bacteria and the type of disease produced. These have been identified in naturally infected horses as well as in experimental infections (29, 30). Age of the host and pathogenicity of the serotype influence the signs. Onset may be peracute, acute, chronic, or the infection may be inapparent and the animal considered a carrier.

Foals less than six months old frequently show the peracute form of the disease. The onset is very rapid and resembles a toxemia or septicemia with or without diarrhea. The mortality rate is very high with death occurring 24 to 72 hours after the foal first appears sick. Joint localization of the infection and arthritis can be sequelae if the animal recovers (1, 3).

The acute form of the disease occurs most frequently in adult horses (2, 3). They may be febrile, weak, anorectic and have liquid feces that may contain blood, mucus, or pieces of the intestinal mucosa. A severe diarrhea can persist in these animals for several weeks with concomitant weight loss and dehydration (2). In milder cases a horse may show only a loss of appetite followed by depression and fever without a severe diarrhea. Although not a consistent finding, neutropenia in the early stages of illness can be helpful in alerting the equine clinician that salmonellosis should be included in the differential diagnosis (3, 29, 31, 32). Patients with the acute form of the disease may die, recover, or become chronic cases.

Horses with chronic salmonellosis may have a persistent diarrhea even though their appetite and temperature are normal. Recovery may occur, but if diarrhea lasts more than five weeks the patient usually dies because of dehydration and weight loss. Chronic cases are quite refractory to therapy and have a grave prognosis (2).

The clinical course of salmonellosis can be influenced by many factors including virulence of the serotype, number of organisms in the inoculum, portal of entry, immune competence of the host, stress, and time of year (10, 33). Examples of stresses are: anthelmintics, change in diet or a dietary deficiency, lack of food or water, strenuous exercise, surgery, shipping, change of stables, antimicrobial therapy or concomitant disease (6, 21). When the host is stressed, the organism multiplies in the gut and is shed in the feces (34, 35). During the summer months more cases of salmonellosis are reported. This may be a result of stress due to management problems rather than weather. Animals

may have inadequate shade, may not have enough water or may be kept in enclosures with insufficient ventilation (5, 10).

### The Carrier State

The carrier state is especially important in the epidemiology of salmonellosis in horses because carrier horses help maintain the infection on isolated farms (3, 6). In surveys of fecal shedders, the less pathogenic serotypes such as agona or anatum have been isolated more frequently. Serotype typhimurium is rarely cultured from asymptomatic carriers except during convalescence. The number of horses shedding the bacteria is related to the region of the country, the season, the population studied, the tissues sampled, and cultural technique used (3).

Some horses are diagnosed as carriers after they die unexpectedly and characteristic postmortem lesions are found along with recovery of the organism (36). Death from salmonellosis often occurs in horses which are referred to veterinary hospitals for surgery. These horses are possibly inapparent carriers and they recover from the surgical procedure but develop acute salmonellosis due to the stress of surgery (3, 6, 21).

Foals born to carrier dams, frequently die from salmonellosis because the mare sheds the organism after the stress of parturition. The offspring is exposed to the organism by suckling from a contaminated udder and from a tendency to be coprophagic (28, 34). A consistent loss of foals may be the only clue that a mare is a carrier; but such a pattern could be overlooked on a large ranch unless careful records are kept and thorough postmortem examinations are performed.

A carrier can be identified by culturing the feces if the horse is shedding the bacteria consistently and in great enough numbers to

be isolated (3, 29, 36). Multiple samples from an individual increase the chance of isolating the bacteria (8). A compromise of between three and five consecutive fecal samples is most frequently used by equine clinicians (3, 8). Demonstration of the bacteria on a herd basis has been more reliable than individual animal culture (10). Some horses with chronic diarrhea were identified as carriers after rectal biopsies were taken (37, 38).

### Antibiotic Therapy

Antimicrobial agents have not been recommended in the treatment of uncomplicated, non-typhoidal salmonellosis because of prolongation of fecal shedding of bacteria without apparent therapeutic effect. Correction of dehydration and electrolyte imbalances have produced a more significant clinical response than antibiotic therapy unless the signs are particularly severe or the animal is septicemic (1, 21, 39). The course of the disease has not been shortened with antibiotics and they are known to prolong the period of convalescent shedding (1, 15, 39).

Also, the presence of bacteria resistant to antibiotics is of great concern. The R factor, a plasmid common to the Enterobacteriaceae, is responsible for resistance to drugs such as tetracycline, kanamycin, chloramphenicol and streptomycin (40, 41, 42). Transferable antimicrobial resistance mediated by R factors has been well documented for salmonella especially for serotype typhimurium (22).

Besides providing no clinical improvement, prolonging the carrier state and potentially introducing multiple drug resistance, antibiotic therapy in horses with salmonellosis disrupts the normal gut flora.

This normal flora competes with salmonella and other pathogens (43). Some mild cases of equine salmonellosis have progressed to acute fulminating septicemias following antibiotic therapy (especially with tetracyclines) (44).

### Sources of Infection

Besides exposure to a carrier, there are many sources of infection for the horse. Vitamin supplements, water and hay grown on fields using contaminated water have been incriminated in outbreaks of salmonellosis (10, 21). Sporadic cases occur more often from direct contact with an infected animal or from contaminated soil while epidemics of salmonellosis are frequently traced to a contaminated food source (16).

Salmonella may remain alive in the soil for long periods of time. Serotype typhimurium survived 120 days in pasture soil and 280 days in well fertilized gardens (26, 30). Other serotypes survived six weeks on vegetation at room temperature and for at least 28 months in dried, naturally infected bird droppings (45). The numbers of bacteria in contaminated soil generally decreased with time, but multiplication can occur if organic material and water are plentiful (46, 47).

### Public Health Aspects of Equine Salmonellosis

Horses occupy a unique position in our culture and are no longer considered just working animals. The majority are companion animals and often have a very close association with their owners. Equine salmonellosis has important public health implications (5, 21). The prevalence of the carrier state in horses is unknown and the number of cases may be underestimated. Many mild cases are missed if culturing

is not performed and if recovery is prompt. Serotype agona has been isolated from horses and from people with gastrointestinal disease (3, 48). Human cases of salmonellosis from contact with pleasure horses have been reported with the majority of cases occurring in veterinarians who had treated infected horses (39, 49, 50). Animals, including horses, are considered to be the greatest reservoir and source of infection for salmonella in man (22).

### Serology

Serological techniques have been used to diagnose salmonellosis in man, cattle and horses and they have saved significant amounts of time in making a diagnosis when compared to fecal cultures. Serology has also been used extensively in the poultry industry to identify carriers of pullorum disease, Salmonella enteritidis bioserotype pullorum and fowl typhoid, Salmonella enteritidis bioserotype gallinarum (51, 52).

Agglutination titers have been used in horses infected with Salmonella enteritidis serotype abortusequi. The presence of antibodies to H antigens were considered to be indicators of recent infections (28, 53). Since this bacteria shares antigens with other members of the Enterobacteriaceae, false positives may occur; however, titers of infected animals are usually much higher than those of noninfected animals (54, 55, 56). A battery of O and H antigens of the most common serotypes of Salmonella enteritidis may help to determine if an organism isolated from a specimen represents a true infection or a contaminant. At least a fourfold rise in titer should be demonstrated during the course of infection (22).

Indirect hemagglutination (IHA), a more sensitive test than the standard tube bacterial agglutination technique, was used to detect antibodies to Salmonella enteritidis serotype dublin in cattle. Infected herds exhibited significantly higher titers than non-infected herds. Herd evaluation was found to be more reliable than individual animal testing (57). Titers greater than 80 in an individual indicated a potential infection. IHA has been useful in determining whether an immune response developed following infection. As with the agglutination test, a rise in titer was necessary to substantiate an active infection (22).

An enzyme-linked immunosorbent assay (ELISA) test has been developed to test mice and rats in commercial laboratories and in research facilities for Salmonella enteritidis serotypes typhimurium, rubislaw, and enteritidis (58). Different degrees of cross reactivity between serotypes were found (59). Typhimurium was the most cross reactive and enteritidis was more cross reactive than rubislaw. Once experimentally infected animals seroconverted, the ELISA test was more sensitive for detecting infected animals than was fecal culture. False positive reactions due to cross reactions were found to be negligible. An ELISA test was evaluated during a human outbreak of salmonellosis in Sweden. Estimation of the increase in titer of antibodies against the O antigen correctly identified 92 percent of the patients who were culturally positive for the organism (59).

Immunofluorescence has been used to identify salmonella in various foodstuffs (60). However, the test was never very accurate due to marked cross reactivity. A new synthetic disaccharide hapten which represents an O antigen coupled to a protein carrier has been developed

(61). Antisera produced from this synthetic hapten has been highly specific when used to identify salmonella by indirect immunofluorescence (IFA). During a large human outbreak (3000 cases) of Salmonella enteritidis serotype enteritidis, IFA was used to screen fecal cultures on enriched media reducing culture time and markedly increasing the sensitivity of the isolation technique (61).

With the development of more sensitive and specific tests and synthetic antigens, serology could be a significant aid in the diagnosis of salmonellosis especially for the carrier animal or when fecal cultures are negative (21). However, antibody evaluation is still an indirect test, requiring the host to respond before a presumptive diagnosis can be made (58). Even if the host responds, there is a delay after infection before antibodies are produced and reach detectable levels.

Serologic techniques including ELISA and IFA, have been used to detect significant antibody titers in animals. There is a great need for such tests to aid in diagnosis of equine salmonellosis and to determine the prevalence of the disease. However, little has been done to evaluate these tests in horses.

#### Vaccines

The use of salmonella bacterins in horses began with the isolation and identification of serotype abortusequi (6, 14, 15, 53). These bacterins were used for prevention because immunity was brief and protection lasted for less than a year. Mares were vaccinated during the fourth and ninth months of gestation and then were boosted yearly in the fall (14). Since vaccination was so common and since this serotype is no longer isolated, bacterins have been credited with

eradication of abortusequi in this country (16, 21, 53). The most common bacterins used against salmonellosis in horses were heat-killed and were preferred because it was thought that somatic, or O antigen, was more accessible (14, 62). At present, a controversy concerning the efficacy of salmonella bacterins in horses exists and their use by equine clinicians has declined (6). There have been reports of successful vaccination programs for serotypes other than abortusequi (14, 15).

Favorable results have been noted in mares who were vaccinated with a formalin-killed bacterin prepared from a culture of the serotype typhimurium isolated on a breeding farm. Two doses of the bacterin were given at 90 and 60 days before foaling. Foal heat diarrheas were less severe and the serotype was not isolated from the feces of the mares (15).

Vaccine efficacy in horses is difficult to evaluate in the literature. Many techniques were used to make the vaccines, only small numbers of animals were vaccinated, no controls were used and different serotypes were compared. In much of the literature, the efficacy of the vaccine was based on the clinical impression of the attending veterinarian (14, 15, 63). Hyperimmunization has been evaluated in mice (64, 65). A series of six intraperitoneal injections of Salmonella typhi vaccine failed to protect BALB/c or C57BL mice from as few as 10 virulent organisms of serotype typhimurium. Hyperimmunization has not been well studied in other animals including horses (66).

Live vaccines have been developed; however, the strain to be used for a vaccine must be stable, must not revert to a virulent form and must provide protection (67). Host-adapted serotypes have been inoculated into abnormal hosts in attempts to stimulate immunity without disease (12). Mice were injected intraperitoneally with  $10^7$

organisms of bioserotype pullorum daily for eight consecutive days, combining hyperimmunization and the use of a live vaccine (64). The mice were challenged on day eight with an LD<sub>50</sub> of serotype enteritidis. Livers and spleens were cultured and daily blood counts were made on randomly selected test and control mice. In vaccinated animals, the challenge dose of serotype enteritidis was cleared rapidly from the blood but organisms increased in the liver and spleen until the fifth day post challenge. The numbers then declined in these tissues. Hyperimmunized mice were more resistant to the toxic effects of heat-killed cells and purified lipopolysaccharide (65).

A great deal of recent research has been done on vaccines to protect cattle from serotype dublin (68, 69, 70, 71). Avirulent rough colony mutants have been used successfully as vaccines in calves and have been commercially available for years (72). An appreciation for the amount of work required to identify and test mutants as possible vaccine strains can be gained from one study. Fifty-five different ways to culture Salmonella enteritidis serotype dublin were evaluated, including growth at different temperatures, on various media, and for different lengths of time. Mice were initially used to evaluate the mutant strains for efficacy in the previously mentioned study. However, the vaccines which afforded the best protection for mice did not provide protection for calves in clinical trials. Testing was then repeated on calves. The best protection in calves was provided by entirely different strains than those that protected mice (67).

There are reports of problems associated with the serotype dublin strain 51 commercially available vaccine. Some calves excreted the organism after vaccination and there were a few cases of postvaccination

anaphylaxis (68, 69, 70, 71). Vaccination of cows in order to passively immunize their calves against serotype dublin has also been studied. The results of these studies have not been consistent (72).

Recombinant DNA techniques have been used to produce live salmonella vaccines and will probably account for most of the future vaccines (73). Salmonella typhi, TY21a, a galactose epimerase mutant, has been tested as a live oral vaccine in children in India (74). Reactions to the vaccine occurred in less than one percent of the vaccinees and involved only a slight diarrhea or soft stool for 24 hours or less (12).

A nonpathogenic mutant of serotype typhimurium (Me Aro-Strain) has been used in calves and mice. This strain requires the aromatic metabolites, para-aminobenzoate (to make enterochelin for iron uptake) and para-aminobenzoic acid (to make folate) which are not available in a mammalian host (69, 75). Mice were protected by the strain when challenged using virulent serotype typhimurium (69). Cross serotype protection has also been observed in cattle. Calves vaccinated with serotype dublin had significantly fewer deaths compared to controls after challenge with virulent serotype typhimurium. Similar results occurred when calves were vaccinated with serotype typhimurium and challenged with serotype dublin (69).

#### Pathogenesis

The enterocolitis syndrome caused by nontyphoidal serotypes generally does not last very long and frequently is self-limiting. After ingestion, the bacteria multiply in the small intestine and colon leading to inflammation of the lamina propria and villi (22). The response is primarily by neutrophils, while in cases of typhoid and

paratyphoid fevers, the response is a mononuclear one. The immunity produced by nontyphoidal enterocolitis is short-lived and repeated infections due to the same serotype are common. In a susceptible host almost any virulent serotype can cause enteric fever, although large numbers may be required to produce disease (22). After ingestion, salmonella attach preferentially to villus tips, pass into the lamina propria, multiply, and are engulfed by phagocytes.

The sites of invasion initially show no evidence of cellular damage or inflammation, but neutrophils soon appear, marginate in submucosal vessels, and move into the villus. This suggests that mucosal damage is partially due to the response of the host (27).

A number of animal models have been used to evaluate virulence of a serotype of salmonella. These include mouse intraperitoneal inoculation, rhesus monkey inoculation, the rabbit ileal loop test and guinea pig LD<sub>50</sub> evaluations. The rabbit ileal loop has been the best model for assessing the invasive and fluid-producing ability of serotype typhimurium. Using this model, it was found that virulence differences exist between different strains of a single serotype. For example, strains SR-2 and Thax-1 did not invade the mucosa while W118, TML, SL 1027, LT-7, and PG-41 did, even though the number of bacteria attached to the epithelial cells and within the mucosa were similar. If the bacteria were injected into the general circulation, strains W118 and TML were cultured more frequently from the liver and spleen while strain M206 was rapidly cleared by the RE. Some strains of serotype typhimurium induce fluid secretion. Strain M206 which produced minimal histological mucosal inflammation evoked fluid secretion comparable to the more pathogenic strains W118 and TML. Strains SL 1027,

LT-7 and PG-41 can invade the mucosa and cause extensive inflammation without fluid secretion (27).

Using the rabbit ileal loop model and strain TML of serotype typhimurium, histology of the infection was studied. The majority of the villi were blunted, swollen, hypercellular and were cuboidal to low columnar in shape. The lamina propria was edematous and extensively infiltrated with neutrophils. The goblet cells contained no mucus. The crypts increased in depth and contained numerous mitotic figures. Mucosal blood vessels appeared normal and ulceration was not usually observed (9).

A less virulent strain, SL 1027, produced fewer alterations in the mucosa with only 15 to 30 percent of the villis showing edema, blunted tips and infiltration with neutrophils. The organisms within phagocytes were morphologically intact despite exposure to digestive components within vacuoles. Virulent serotypes have the ability to survive and multiply within the vacuoles (9).

#### Immune Response to Salmonella

Although vaccines from killed organisms increase the resistance to salmonellosis, a more solid immunity develops from natural infection or vaccination with live bacteria (10). Specific antibodies increase phagocytosis by opsonization; however, a humoral response to serotype enteritidis in mice has been demonstrated to occur without any resistance to challenge infection (64, 65, 76, 77). The amount of protection that serum has been able to provide by passive immunization depended on the route of administration and time between immunization and challenge (78).

Since pathogenic salmonella can resist phagocytosis, effective resistance is primarily due to cell mediated immunity as for most facultative intracellular bacteria (65, 79). Effective vaccines must stimulate CMI to be effective but humoral immunity may or may not occur. In studies using calves, a Salmonella enteritidis serotype dublin vaccine provided protection against challenge from a virulent strain without a marked antibody response. Agglutination titers were low and did not correlate with protection (68).

#### Skin Testing in Horses

Skin testing for diseases such as tuberculosis and glanders has been used in horses. The mallein test for glanders uses antigens prepared from media on which the bacteria has been grown. The antigen is injected intradermally or subcutaneously into the palpebrae. The test is read in 72 hours and swelling around the eyes indicates a positive response (80, 81).

One study evaluated the tuberculin test in horses intended for slaughter and found no significant correlation between the intradermal test response, findings at postmortem, and isolation of pathogenic Mycobacteria (82). Reasons for the poor response to the tuberculin skin test were unknown. The response may have been due to the nature of the test or to other factors such as a low infection rate. Skin testing has not been performed as an aid in diagnosing cases of equine salmonellosis.

The correlation between serum immunoglobulin levels, lymphocyte transformation studies and dermal responses in horses with chronic diarrhea was studied (83). No etiologies were given for the diarrheas.

The results indicated that the serum concentration of immunoglobulin G (IgG) was greater in sick horses. However, lymphocytes showed a transient depression in their response to phytohemagglutinin A (PHA), in transformation studies and the intradermal skin tests using antigens from common horse feeds were significantly less than in normal horses. The horse in this study appeared to be immunosuppressed (83).

Skin testing with PHA has been used to identify horses with combined immunodeficiency and resultant inability to mount a cell mediated immune response (84, 85). Fifty micrograms of PHA can be injected intradermally to evaluate the ability of the horse to mount a cell-mediated immune response (83, 86, 87). After intradermal injection, there is a noticeable swelling within four hours that peaks between 24 and 72 hours. Histological examination of biopsies at the injection site shows primarily a mononuclear infiltrate although mast cells were also present in normal horses. The combined immunodeficient Arabian foals have a reaction that peaks at four hours and then steadily declines (84, 85).

#### Control

Since salmonella can survive for long periods in the soil, good animal management and husbandry techniques could help reduce exposure from environmental contaminants. Identification and isolation or euthanasia of carrier horses would also help reduce infection in susceptible horses and environmental contamination. Pasture rotation could reduce the incidence of salmonellosis on certain property (45, 46, 47). Standing water should be drained. No other animals should be allowed to graze on infected pasture and forage crops should not be

grown on contaminated land. Carcasses or any source of organic material should be removed in order to reduce the potential for multiplication of the organism (10). Control and prevention of salmonellosis is extremely difficult because the bacteria is so hardy and widespread in nature (45).

## CHAPTER III

### MATERIALS AND METHODS

#### Experimental Animals

Three experiments were performed using five infected horses and two controls. Experiment I involved, horse A, a two year old, quarterhorse filly which was shedding Salmonella enteritidis serotype braenderup. Isolation of the bacteria was made by the microbiology laboratory at the veterinary school and was typed by the National Veterinary Services Laboratory at Ames, Iowa.

A two year old, quarterhorse filly, horse B was used in experiment II. She was shedding serotype agona in her feces. Both horses were kept in isolation stalls which were approximately 14 square meters.

The third experiment involved three privately owned yearling quarterhorses, two females, C and D, and a castrated male, E. They were from the same ranch as horse B and were also shedding serotype agona. After these horses were found to be shedding the bacteria, they were isolated from other horses and kept on grass pasture. An autogenous bacterin (Bio-Med Research Laboratories, Seattle, Washington) had been prepared for this farm from an isolate of serotype agona. Horses C, D and E had received a series of three vaccinations of this commercial preparation given at two week intervals six months before they were involved in this research.

Two horses, F and G, which were culturally negative for salmonella were used as controls in experiments II and III. Horse F was a five year old Appaloosa stallion and horse G was a three year old quarterhorse gelding.

#### Fecal Culture

All media used were obtained from Difco Laboratories, Detroit, Michigan and reagent grade chemicals were used to prepare the phosphate buffered saline (PBS).

Salmonella were isolated from feces using enrichment techniques. For horse A, 10 ml of PBS was added to 10 g of feces, macerated, then one loopful was used to streak a MacConkeys and brilliant green agar plate. One ml of the diluted feces was added to 10 ml of selenite broth and the remainder of the diluted feces was added to 100 ml of tetrathionate broth. Subcultures from the enrichment broths were made on brilliant green and Salmonella-Shigella agar. A different protocol was used for the other horses (90). One ml of sterile PBS was added to 30 g of feces in a sterile polypropylene tissue collection bag. This was placed in a second bag, then macerated for one minute using a Stomaker (Tekmar Co., Cincinnati, Ohio). A loopful was used to directly streak MacConkeys and brilliant green agar plates for isolation. One ml of the diluted feces was added to 10 ml of selenite broth and incubated at 37 C, and the rest of the feces were added to 100 ml of tetrathionate broth and put in a tissue collection bag along with one to two mls of iodine to retard the growth of other enteric bacteria. The bag was placed in a container to give it support and incubated at 40 C. The enrichment broths were incubated for 24 hours then subcultured to

MacConkeys and brilliant green agar plates by streaking the plates. The enriched fecal samples were then reincubated for a second 24 hours and subcultured a second time. Plates were incubated for 24 hours, then observed. After biochemical and polyvalent O antiserum testing, a positive isolate was evaluated using an API-20 E (Analytical Products, Plainview, New York) and if identified as a salmonella, was sent to the National Veterinary Services Laboratory in Ames, Iowa for serotyping.

Feces from horses A and B were cultured twice during the experiment and at postmortem. Feces were collected from horses C, D, and E three times: before, during and after the vaccination series in experiment III. Five consecutive fecals from the control horses were collected and cultured for salmonella. Both control horses were found to be negative before they were used in the experiment.

#### Antigen Preparation

In order to grow large numbers of the organisms, eight ounce prescription bottles were filled with a thin slant of brain heart infusion (BHI) agar. Serotypes were inoculated into three ml of BHI broth, vortexed and then 0.5 ml was used to inoculate each of six prescription bottles. Following an 18 hour incubation, bacteria were harvested from the bottles in three ml of PBS by periodic rocking for one hour. The bacteria-rich saline was transferred to sterile vaccine bottles and the bacteria were killed by adding 0.6 percent formalin-saline solution sufficient to make a McFarland tube three concentration (91, 92). Inactivation of the bacteria with formalin preserves the H antigens (93).

The formalin-killed vaccine prepared for horses C, D, and E was also heated in a waterbath at 60 C for one hour to ensure that the organism was killed. The antigen preparations were tested for sterility after two days of room temperature incubation by inoculating three tubes of fluid thioglycolate with 0.1, 0.5, or one ml of antigen. The tubes were incubated at 37 C and observed for growth at 24 and 48 hours. Some of the bacterial suspension of serotypes agona and braenderup was not diluted. Instead, it was killed with one ml of 40 percent formaldehyde solution and tested for sterility as previously described except that only 0.1 ml of the antigen was inoculated into fluid thioglycolate. Dilutions for skin testing were made from this concentrated antigen and used to evaluate the sensitivity of each animal to the antigen so that the best antigen dose would be used during vaccination.

O antigens were prepared using the following technique. Bacteria were grown and harvested as described for the H antigen. The bacterial suspensions were collected and placed in sterile 100 ml vaccine bottles with sufficient PBS to give a McFarland three turbidity. These bottles were placed in a water bath at 60 C for one hour then sterility was checked using the same method as described previously and the antigens were stored at 5 C (91, 92, 93).

Ten antigen dilutions were prepared from heat-killed serotype agona and were used in the Macrophage Inhibition Factor (MIF) and Lymphocyte Stimulation (LST) tests. Serial, two-fold dilutions of the organism were made in RPMI-1640 (Gibco, Grand Isle, New York). Two ml aliquots of the antigens were then placed in screw-top, sterile plastic tubes and kept at 5 C until the morning of a test.

The serotype agona isolated from horse B in experiment II was also used to prepare H and O antigens used in experiment III. Similar

preparations were made from serotypes newport, typhimurium, heidelberg and anatum by the previously described techniques. Each of these serotypes represented a group classification according to Ewing's abbreviated antigen scheme (94). These antigens were compared to the infecting serotypes using the bacterial agglutination test.

#### Endotoxin Evaluation of Vaccines

The H antigen, formalin-killed vaccines were tested for endotoxin using the Limulus Amoebocyte Lysate test (LAL) (M. A. Bioproducts, Walkersville, Maryland) according to the manufacturers directions. Known concentrations of E. coli endotoxin were compared to dilutions of the sample to be tested. Three concentrations of the serotype braenderup and agona vaccine were tested; the McFarland three concentration, a 1:2 dilution and a 1:4 dilution.

#### Bacterial Agglutination Test

Antibody was titrated by the microtiter method. Roundbottomed, 96 well microtiter plates (Titertek Microliter Plates, Flow Labs, Rockville, Maryland) were used (95).

Two-fold serial dilutions of an initial 1:10 dilution of serum were made in PBS so that each well contained 100 microliters. An equal volume of antigen was added to each well. One well containing only antigen and PBS was used as a control. The plates were covered and incubated at 37 C overnight and read the next morning using reflected light. The titer was the reciprocal of the last well in which agglutination was observed (95).

### Skin Tests

Horses were prepared for skin testing by shaving the neck with an electric clipper and then cleaning the skin with 70 percent alcohol which was allowed to air dry. The antigen was injected in 0.1 ml volumes intradermally with a tuberculin syringe and a 26 gauge, 3/8 inch needle (Becton Dickinson and Company, Rutherford, New Jersey) on the lateral aspect of the neck between the angle of the jaw and the shoulder (82, 83, 84, 85, 89). The day that skin testing was performed was designated as day one for all experiments. The injection sites on horses A and B were checked at 2, 4, 24, 48 and 72 hours and at 24, 48, and 72 hours in horses C, D, and E. Horse A was tested with serotype braenderup antigen and horses B, C, D, and E were tested with serotype agona antigen. A PBS control was omitted in horse A, but used for testing the other horses.

### Pathology

Horses A and B were euthanized on day 111 and on day 115 of the experiments, respectively, and postmortem examinations and fecal cultures were performed. In addition, liver, spleen, kidney and the area of skin which received the undiluted antigen were collected from horse B and submitted for histological examination.

### Immunization Schedule

A week was allowed between skin testing and the first vaccination. Initially, the horses were injected subcutaneously to observe potential vaccination reactions. Later, injections of gradually increasing volumes of vaccine were given intramuscularly. Horses A and B were

vaccinated biweekly for six consecutive weeks with serotype braenderup and agona respectively. Horses C, D, and E were vaccinated with serotype agona. The second of the biweekly vaccinations for these horses was given by the ranch manager. If any animal showed a marked reaction to the injection, a second injection was not given that week.

#### Blood Collection

Following preparation of the skin with 70 percent alcohol, 10 mls of blood were collected from the jugular vein into clot tubes (Vacutainer, Becton Dickinson, Rutherford, New Jersey). The blood was allowed to clot at room temperature, centrifuged at low speed for five minutes and the serum removed and stored at 0 C until used. Blood for the collection of white cells was drawn into 10 ml preservative free sodium heparin tubes (Vacutainer) and transported to the lab where it was maintained at room temperature with mechanical rocking. Cell harvesting was begun within three hours after collection. On hot days, tubes were transported in a styrofoam cooler with cold but not frozen ice packs. This was to protect the blood during transport without altering metabolism and microtubule formation (96).

#### White Blood Cell Harvest

Two different techniques for harvesting white blood cells were evaluated. The first was an osmotic lysis of the red blood cells (RBC's), originally developed for bovine blood (97, 98). This method resulted in harvests of lymphocytes, granulocytes, monocytes and residual RBC's and was used only in experiment II. Eighty mls of sterile distilled water at room temperature was added to 40 ml of

heparinized blood in a sterile plastic graduated cylinder (Corning, Corning, New York) and mixed by inversion of the capped cylinder. Exposure to the hypotonic solution was lengthened from an initial time of 30 seconds to 60 seconds because too many RBC's remained. Isotonicity was restored by adding 40 ml of sterile, phosphate-buffered (0.013 M) double-strength NaCl (1.7 percent solution) at a pH of 6.8. Gentle inversion was used to mix the suspension, which was then transferred to sterile centrifuge tubes (Corning) and centrifuged at 220 g for eight minutes. The supernatant was removed by vacuum aspiration and the cells were resuspended in Eagle's Minimum Essential Medium, (EMEM) (Gibco). Sodium heparin (Gibco), was added to give a concentration of five units per ml. A vortex mixer at a low setting was used to gently disperse the pellet of white blood cells in the tissue culture media. The suspension was again centrifuged at 220 g for six minutes, the supernatant was aspirated and discarded and the cells were resuspended in EMEM that contained 50 micrograms per ml of gentamicin reagent solution and 10 percent sterile, fetal bovine serum (FBS). The cells were washed twice using the above procedure. In some experiments 10 percent donor horse serum (Gibco) was used instead of FBS.

The second technique involved cell separation on a concentration gradient of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, New Jersey) which has been adapted for horses (99). Thirty mls of heparinized blood were diluted with an equal volume of sterile PBS. Between 10 and 12 ml of the diluted blood was carefully layered on 10 ml of Ficoll-Paque. The tubes were centrifuged at 1800 g for 20 minutes at room temperature. The layer of mononuclear cells, present at the plasma RBC interface, was removed with a sterile pipette. The

cells, approximately five ml in volume, were washed twice using 20 ml of PBS and centrifuged at 1800 g for 10 minutes at room temperature. After the second wash the pellet was suspended in three ml of RPMI-1640 with 100 micrograms of gentamicin sulfate and 20 percent donor horse serum.

#### Viability Measurement

The trypan blue dye exclusion test was used to evaluate cell viability (98). A drop of cell suspension was mixed with a drop of trypan blue, 0.4 percent concentration (Gibco) on a glass slide. One hundred cells were counted and the percent viability was determined.

#### Standardization of the Cell Suspension

The number of mononuclear cells per ml in the tissue culture media-cell suspension was determined using the Unopette Test (Becton Dickinson). Cells were counted in a Neubauer improved hemocytometer. The total number of white blood cells was determined by counting the four large corner squares of both chambers, dividing by two and multiplying by 50,000 to give cells/ml. The concentrated cell suspension was then diluted with RPMI-1640, which contained gentamicin sulfate and donor horse serum, to give a final concentration of 100,000 cells/100 microliters (97).

#### Leukocyte Migration Inhibition Test

The procedure of Dorsey, developed for studying Brucella abortus in cattle, was used for the leukocyte migration inhibition (LMI) test (97). Agarose (Oxoid, London, England and Seakem, SMC Corporation,

Rockville, Maryland) two percent in distilled water was steamed for 20 minutes then held in a rocking water bath at 48 C until used. Double strength Eagle's Minimum Essential Medium (2x EMEM) was prepared. The 2x EMEM was filter-sterilized (Nalgene, Nalge Co., Sybron Corp., Rochester, New York) and gentamicin sulfate (final concentration 20 percent) were added to the solution which was then equilibrated at 48 C. The agarose and EMEM solutions were mixed together under a laminar flow hood. Plates were prepared by pouring 7 ml of the agarose-tissue culture medium into sterile 60 mm by 15 mm tissue culture plates. When the plates solidified six wells were cut, each measuring 4 mm in diameter and 10 mm apart by use of a template. A sterile Pasteur pipette and vacuum system were used to aspirate the agarose from the wells.

Cells collected by the flash lysis technique were not counted in Dorsey's technique, because they clumped, but were mixed well and divided into two equal volumes in separate tubes before the third wash. The cells in each tube were then resuspended in 50 microliters of EMEM containing 10 percent FBS and 50 micrograms/ml of gentamicin sulfate. One tube served as the control and the second received 50 microliters of antigen. The cell suspensions were incubated in a shaking water bath at 37 C for 90 minutes, vortexed gently to resuspend the cells at the end of the incubation period, and then used to fill the wells of the agarose plates. The plates were then incubated at 37 C for 24 hours in an incubator with 95 percent air saturated with water vapor and five percent CO<sub>2</sub>. Following incubation, the plates were flooded with 10 percent formalin for 20 minutes, the agar was removed, and the cells were stained with Wright-Giemsa stain (Volu-Sol,

Medicare Industries Inc., Las Vegas, Nevada). The plates were then rinsed with distilled water and air dried. The diameters of the migration zones were measured using an ocular micrometer (American Optical, Buffalo, New York). In some of the tests donor horse serum was substituted for the FBS in the medium and cells were collected by density centrifugation.

#### Lymphocyte Stimulation Test

Lymphocytes were harvested by differential centrifugation on Ficoll-Paque as previously described for mononuclear cell collection. The cells were standardized and a concentration of 100,000 cells per 100 microliters per well was used for the lymphocyte stimulation test. One hundred microliter aliquots of the cell suspension were placed in a 96 well tissue culture plate (Costar, Cambridge, Massachusetts) using an automatic pipette (Finnpipette, Lab Systems, Helsinki, Finland).

Antigens 1 through 10, prepared as described before, were added in 100 microliter amounts. Controls consisted of 100 microliters of tissue culture media with antibiotic and horse serum. All antigens, mitogens and the controls were run in triplicate except as noted in the results.

The following mitogens were reconstituted according to manufacturers recommendations and used in the LST test: Bacto Phytohemmagglutinin, PHA (Difco), Bacto Concanavalin A, Con A (Difco), and Pokeweed mitogen PWM (Gibco). The Con A was made up to five mg/ml and the PWM four mg/ml; the activity for the PHA was unknown by the manufacturer but rehydrated according to directions. The mitogens were diluted 1:5, 1:10, and 1:20 in RPMI-1640 for use in the test. These dilutions were designated respectively as 1, 2, and 3. The covers were then taped on and the

plates were incubated at 37 C in a five percent CO<sub>2</sub> incubator with 95 percent air and water vapor for 72 hours. At the end of this time 20 microliters of tritiated thymidine, prepared by the addition of five ml of methyl-<sup>3</sup>H thymidine, five microcuries per millimole (ICN, Irvine, California) to 120 ml of RPMI-1640 containing 50 micrograms/ml of gentamicin sulfate was added to each well. After an additional incubation period of 18 hours the cells were killed by putting the plates into the freezer compartment of a standard, frost-free refrigerator until frozen. The plates were thawed and the cells were harvested onto glass filter paper discs using a cell harvester (Titertek Cell Harvester, Flowlab, McLean, Virginia).

The paper was allowed to dry at room temperature for several hours, then the discs were placed in two mls of scintillation fluid [Bis-MSB (two percent) and PPO (98 percent) premixed scintillation powder, ICN] and read in a scintillation counter (Packard Tri-Carb 300, Packard Instrument Co., Downersgrove, Illinois). The level of <sup>3</sup>H-thymidine uptake was measured and the stimulation index was calculated (94, 98).

#### Data Analysis

A computer program was written so that the scintillation counts could be entered on the main frame computer at the university. The only statistic performed was the determination of the means of the counts per minute. The stimulation index was calculated by the standard procedure (96, 100).

## CHAPTER IV

### RESULTS

Salmonella enteritidis serotype braenderup was isolated twice from horse A during experiment I in fecal samples on days 22 and 59. However, the organism was not recovered from the mesenteric lymph nodes and the distal small intestine when horse A was euthanized on day 111. In experiment II, serotype agona was isolated from mesenteric lymph nodes as well as from the distal small intestines of horse B at necropsy; however, two fecal samples collected during the experiment were culturally negative. Although serotype agona was also isolated from horses C, D, and E prior to beginning the experiment, fecal samples collected twice during experiment III were culturally negative. Fecal samples were collected each day for five days from control horses F and G before they were used in this study. Culturing by the previously described methods resulted in no isolation of salmonella.

The H antigen preparations of serotype agona and braenderup were used as vaccines. Endotoxin was evaluated in these preparations using the LAL test. The McFarland three concentrations and a 1:2 PBS dilution of both serotypes showed a positive reaction to the LAL test indicating endotoxin at the level of 0.25 ng/ml. The 1:4 dilution of the vaccines were negative.

Serotype braenderup was used to skin test horse A and serotype agona was used to skin test horses B, C, D, and E. The purpose of the

skin test was two-fold. Sensitivity to the antigen was determined and the appropriate concentration of vaccine to use was decided from the results of this test. In addition the delayed-type hypersensitivity response to the salmonella antigen was evaluated by the presence of a reaction 72 hours after injection (79). The prevaccination skin test results for horse A were: a reaction to the undiluted and a 1:4 dilution of the skin test antigen. Post-vaccination results were similar except that there was also a reaction to the 1:16 dilution of antigen (Table I). Horse B had a prevaccination reaction to the undiluted, and a minimal reaction to the 1:20 dilution. A slight swelling was noted at all injection sites at four hours. The undiluted and 1:20 antigen dilutions resulted in firm, 2 cm, circumscribed reactions that were gone within an additional four days. The postvaccination reaction to the undiluted antigen resulted in frank necrosis at the injection site and swelling of the entire ipsilateral side of the neck. Seventy-two hours after the injections were given all the antigen skin test reactions were less than 2 cm in diameter except for the undiluted site which was larger and contained a necrotic center with a scant amount of thick, yellow-white exudate (Table II).

Undiluted antigen was not used to test horses C, D, and E because of the reaction of horse B and only prevaccination skin tests were performed. Twelve hours after injection, horses C, D, and E had reactions at the 1:20, 1:40, and 1:80 dilutions. After 72 hours, horses C and D had reactions to the 1:20 dilution (Table III). Turbidity was noted at 72 hours in the fluid thioglycolate tube used to test the skin test antigens of horses C, D, and E. The antigen was diluted and used when the 48 hour sterility test showed no growth. Inadvertently, a

TABLE I. Skin test results for horse A.

Dilution**	Reactions*	
	Day 3	Day 11
Undiluted	++	++
1:4	++	++
1:16	-	++
1:64	-	-
1:256	-	-
1:1024	-	-

\* Skin test performed on day 0 and evaluated 72 hours later.

\*\* Serotype braenderup antigen in PBS dilutions.

++ 2 cm circumscribed swelling.

- No reaction.

TABLE II. Skin test results for horse B.

Dilution**	Reactions*	
	Day 3	Day 115
Saline Control	-	-
Undiluted	++	++++
1:20	+	+
1:40	-	+
1:60	-	-

\* Skin test performed on day 0 and evaluated 72 hours later.

\*\* Serotype agona antigen in PBS dilutions.

<sup>+</sup> Less than 2 cm, circumscribed swelling.

<sup>++</sup> 2 cm, circumscribed swelling.

<sup>++++</sup> 4 cm, frank necrosis.

<sup>-</sup> No reaction.

TABLE III. Skin test results for horses C, D, and E.

Dilution**	Reactions*		
	Horse C	Horse D	Horse E
Saline Control	-	-	-
1:20	+	+	+/-
1:40	-	-	-
1:80	-	-	-

\* Skin test performed on day 0 and evaluated 72 hours later on day 3.

\*\* Serotype agona antigen in PBS dilutions.

+ Less than 2 cm, circumscribed swelling.

+/- Palpable but not measurable swelling.

- No reaction.

culture that was not killed was used to skin test the horses in experiment III. With the exception of horse D, which developed a transient diarrhea, there were no clinical signs and all the horses were bright, alert and did not go off feed.

The McFarland three dilution of the formalin-killed preparation which preserved the H antigens was used as the vaccine. This standard was approximately equal to a dilution of 1:80 on the skin test and was used because no horse demonstrated an initial skin reaction to this dose after 72 hours. The infected horses were vaccinated biweekly for a period of up to seven weeks. The vaccination schedules are given in Tables IV-VI. Horse B was not vaccinated on day 100 of experiment II, when she was found down in her stall, unable to rise (Table V). Horses C, D, and E had vaccination reactions to the dose given on the 15th day of experiment III. All demonstrated marked swelling of the neck in the region of the vaccination site. The animals were rested for one week and a reduced volume of vaccine was given (Table VI). In addition, the stools of horse D were loose for several days immediately following vaccination throughout the experiment. On day 36, horse E developed swollen hocks after the injection was given. However, the animal was never lame and showed no other clinical signs while the swelling gradually decreased.

The results of the bacterial agglutination tests in experiments I, II, and III were as follows: horse A had titers to O and H antigen preparations of serotypes braenderup, heidelberg, newport and anatum. The H antigen titers were consistently higher than the O antigen titers and the highest titers were to the H antigen preparation of the infecting serotype, braenderup (Table VII).

TABLE IV. Vaccination schedule for horse A.

Day of Experiment*	Volume
4	0.5 ml**
8	1.0 ml**
11	1.0 ml
15	1.5 ml
18	2.0 ml
22	2.0 ml
28	3.5 ml
31	4.0 ml
35	4.0 ml
38	2.0 ml
42	5.0 ml+
44	5.5 ml+
49	5.5 ml+
52	6.0 ml+

\*Serotype braenderup used as antigen.

\*\*Injections given subcutaneously, all others given intramuscularly using different muscle masses.

+Volumes greater than 4.0 ml were divided and given into two sites.

TABLE V. Vaccination schedule for horse B.

Day of Experiment*	Volume
65	1.0 ml**
69	1.5 ml**
72	2.0 ml
76	2.0 ml
79	3.0 ml
84	4.0 ml+
87	4.0 ml+
94	5.0 ml+
98	5.5 ml+
101	6.0 ml+
105	6.0 ml+

\* Serotype agona used as antigen.

\*\* Injections given subcutaneously, all others given intramuscularly using different muscles masses.

+ Volumes greater than 4.0 ml were divided and given into two sites.

TABLE VI. Vaccination schedule for horses C, D,  
and E.

Day of Experiment*	Volume
8	0.5 ml**
12	1.0 ml**
15	1.5 ml**
21	1.0 ml**
29	1.0 ml
33	1.5 ml
36	2.0 ml
50	2.0 ml
54	2.0 ml

\* Serotype agona used as antigen.

\*\* Injections given intramuscularly using  
different muscle masses.

TABLE VII. Salmonella agglutination titers for horse A.

Serotypes	Group**	Days Post Vaccination*	
		0	55
braenderup <sup>+</sup>	C <sub>1</sub>		
H antigen		20,480	327,680
O antigen		2,560	20,480
heidelberg	B <sub>1</sub>		
H antigen		80	640
O antigen		20	320
newport	C		
H antigen		40	160
O antigen		10	20
anatum	E		
H antigen		2,560	20,480
O antigen		40	320

\* Two fold serial dilutions of a 1:10 serum dilution using a 96 well microtiter plate.

\*\* Ewing's abbreviated antigen scheme (94).

<sup>+</sup> Serotype infecting horse A.

Titers to all serotypes increased after vaccination; however, the most significant increase was to braenderup. Horses B, C, D, and E, infected with serotype agona, all had prevaccination titers to serotypes agona, newport, heidelberg, and anatum. The titers increased to all serotypes during vaccination; but, the highest titers were still to the H antigen preparation of the infecting serotype (Tables VIII-IX). Horses F and G, the controls, had titers to serotypes agona, heidelberg, anatum and newport, with the highest titers to the H antigen preparations. Horse F had the highest titer to serotype anatum and horse G had the highest titers to serotypes agona and anatum (Tables X-XI).

White blood cells harvested by the flash lysis technique were not counted because problems with clumping prevented an accurate count. White blood cells collected by differential centrifugation on Ficoll-paque, belonged to the mononuclear series and were counted. Their numbers varied from week to week and from animal to animal. Horse B had a range of 15,800 to 17,600 cells/ml after differential centrifugation and resuspension in tissue culture media. Horse C ranged from 33,200 to 34,800 cells/ml; horse D, 25,600 to 55,800 cells/ml; and horse E, 22,500 to 38,600 cells/ml. The control horses also demonstrated similar variability with horse F having between 18,700 and 20,600 cells/ml and horse G having between 13,000 and 42,000 cells/ml.

Cell viability, measured by trypan blue dye exclusion was far more consistent and ranged between 95 and 100%. The average viability was 98% for all samples tested.

Results of the leukocyte migration inhibition test were variable. Migration was observed on only one occasion. Despite the substitution of Donor Horse Serum for FBS and Sea Kem Agarose for Oxoid agarose, as

TABLE VIII. Salmonella agglutination titers for horse B.

Serotypes	Days Post-Vaccination*								
	1	8	15	22	29	36	43	50	57
heidelberg									
H antigen	80	80	100	160	320	640	320	640	640
O antigen	40	80	80	80	80	160	80	160	160
agona**									
H antigen	2,560	20,480	20,480	40,960	81,920	81,920	163,840	327,680	327,680
O antigen	20	40	80	80	160	320	320	640	640
newport									
H antigen	40	40	40	80	80	80	80	160	320
O antigen	20	20	20	40	40	40	40	80	80
anatum									
H antigen	160	320	320	320	320	320	320	640	640
O antigen	40	160	160	160	160	160	160	160	160

\* Two-fold serial dilutions of a 1:10 serum dilution using a 96 well microtiter plate.

\*\* Serotype infecting horse B.

TABLE IX. Salmonella agglutination titers for horses C, D, and E.

Day of Experiment Horse		Serotypes*							
		heidelberg		agona**		newport		anatum	
		Antigens							
		H	O	H	O	H	O	H	O
1	C	80	40	1,280	80	320	40	1,280	40
	D	80	40	640	40	640	40	320	40
	E	40	20	10,240	80	80	40	320	80
8	C	160	80	10,240	80	640	40	1,280	80
	D	160	80	1,280	80	320	40	640	80
	E	80	40	40,960	80	320	80	640	80
15	C	160	40	10,240	80	640	40	2,560	80
	D	80	40	2,560	80	320	40	640	80
	E	80	40	81,920	160	640	80	640	80
22	C	320	80	10,240	80	1,280	80	2,560	80
	D	160	80	10,240	160	320	80	320	80
	E	80	40	40,960	80	320	80	640	80
36	C	320	80	20,480	80	640	80	1,280	80
	D	160	40	10,240	160	640	160	640	80
	E	80	40	81,920	160	640	80	640	80
43	C	320	80	20,480	80	1,280	80	1,280	80
	D	160	80	10,240	160	640	160	640	80
	E	80	40	163,840	160	640	80	640	80

\* Two-fold serial dilutions of a 1:10 serum sample in a 96 well microtiter plate.

\*\* Serotype infecting horses C, D, and E.

TABLE X. Salmonella agglutination titers for control horses F and G, experiment I.

Day of Experiment	Horse	Serotypes*							
		<u>heidelberg</u>		<u>agona</u>		<u>newport</u>		<u>anatum</u>	
		Antigens							
		H	O	H	O	H	O	H	O
1	F	40	20	160	40	80	40	320	80
	G	40	10	160	40	80	40	160	40
8	F	40	20	160	80	80	40	320	80
	G	40	20	160	40	80	40	160	40
15	F	20	10	160	40	160	40	320	40
	G	40	20	160	80	80	40	160	40
22	F	40	20	160	80	80	40	320	40
	G	40	20	160	40	80	20	160	40
36	F	40	10	160	40	80	40	320	80
	G	40	20	160	80	80	40	160	80
43	F	40	20	160	80	80	40	320	80
	G	40	20	160	40	80	20	160	40

\* Two-fold serial dilutions of a 1:10 serum dilution in a 96 well microtiter plate.

TABLE XI. *Salmonella* agglutination titers for control horses F and G, experiment II.

Day of Experiment	Horse	Serotypes*							
		heidelberg		agona		newport		anatum	
		H	O	H	O	H	O	H	O
1	F	40	20	320	80	160	40	640	160
	G	40	20	320	80	160	40	160	40
36	F	20	10	160	80	160	40	320	80
	G	40	20	320	80	160	40	80	40
43	F	40	20	320	40	80	40	320	80
	G	40	20	640	80	160	40	160	40
50	F	40	20	160	40	80	40	320	80
	G	40	20	320	80	160	40	160	40
57	F	40	20	160	80	80	40	160	80
	G	40	20	320	80	160	40	80	40

\*Two-fold serial dilutions of a 1:10 serum dilution in 96 well microtiter plate.

well as a change to cell collection by density gradient centrifugation, the test did not produce positive results and was abandoned. The lymphocyte stimulation test (LST) was used instead to evaluate CMI. The level of three H-thymidine uptake was measured in a liquid scintillation counter and the stimulation index was calculated (96, 100).

The mitogen dilutions which gave the highest SI results for control horse G were: PWM dilution two on day one (3.93) and PHA dilution two on day one (6.01), of experiment III.

Results of the LST test were variable; however #6 dilution of serotype agona antigen produced the most significant SI compared to other dilutions (Tables XII and XIII). When SI and days after vaccination were graphed for antigen #6, test horse B did not have significantly greater SI than the control horses F and G in experiment I (Figure 1). However, in experiment II the mean SI for the three test horses, C, D, and E, was greater than the mean of the SI for the two control horses, F and G (Figure 2).

At the end of experiment I, horse A was euthanized and a gross postmortem examination was performed. The horse had gained several hundred pounds during the experiment and no significant postmortem changes were noted. Tissues were not examined histologically. Animal B was euthanized on the 115th day of the second experiment. Ascarids were noted at postmortem examination but no remarkable gross changes were noted. Histopathology of the kidney revealed aggregates of lymphocytes, plasma cells and macrophages in the interstitial cortical area (Figure 3). Tufts of some glomeruli were adherent to Bowman's capsule and there was a variation in size between glomeruli. There was only a scant amount of material in the tubules and damage to the kidney was

TABLE XII. Lymphocyte blast transformation data\*, serotype agona antigen #6--experiment I.

Animals	Days After Initial Vaccination						
	8	15	22	29	36	43	50
B	1.69	-- <sup>+</sup>	--	--	1.21	1.25	--
F <sup>++</sup>	--	2.70	--	NA**	--	1.77	--
G <sup>++</sup>	NA	NA	--	NA	2.50	2.53	1.16

\*SI =  $\frac{\text{mean of counts per minute for triplicate stimulation culture}}{\text{mean of counts per minute for triplicate control cultures}}$ .

<sup>+</sup>SI less than one.

<sup>++</sup>Control horse.

\*\*Not available.

TABLE XIII. Lymphocyte blast transformation data\*, serotype agona antigen #6--experiment II.

Animals	Days After Initial Vaccination				
	8	15	22	36	43
C	4.27	-- <sup>+</sup>	--	--	1.13
D	--	--	--	6.31	--
E	1.18	--	--	25.27	1.98
F <sup>++</sup>	--	--	--	--	4.07
G <sup>++</sup>	--	--	--	2.59	--

\*SI =  $\frac{\text{mean of counts per minute of triplicate stimulated cultures}}{\text{mean of the counts per minute of triplicated control cultures}}$ .

<sup>+</sup>SI less than one.

<sup>++</sup>Control horse.

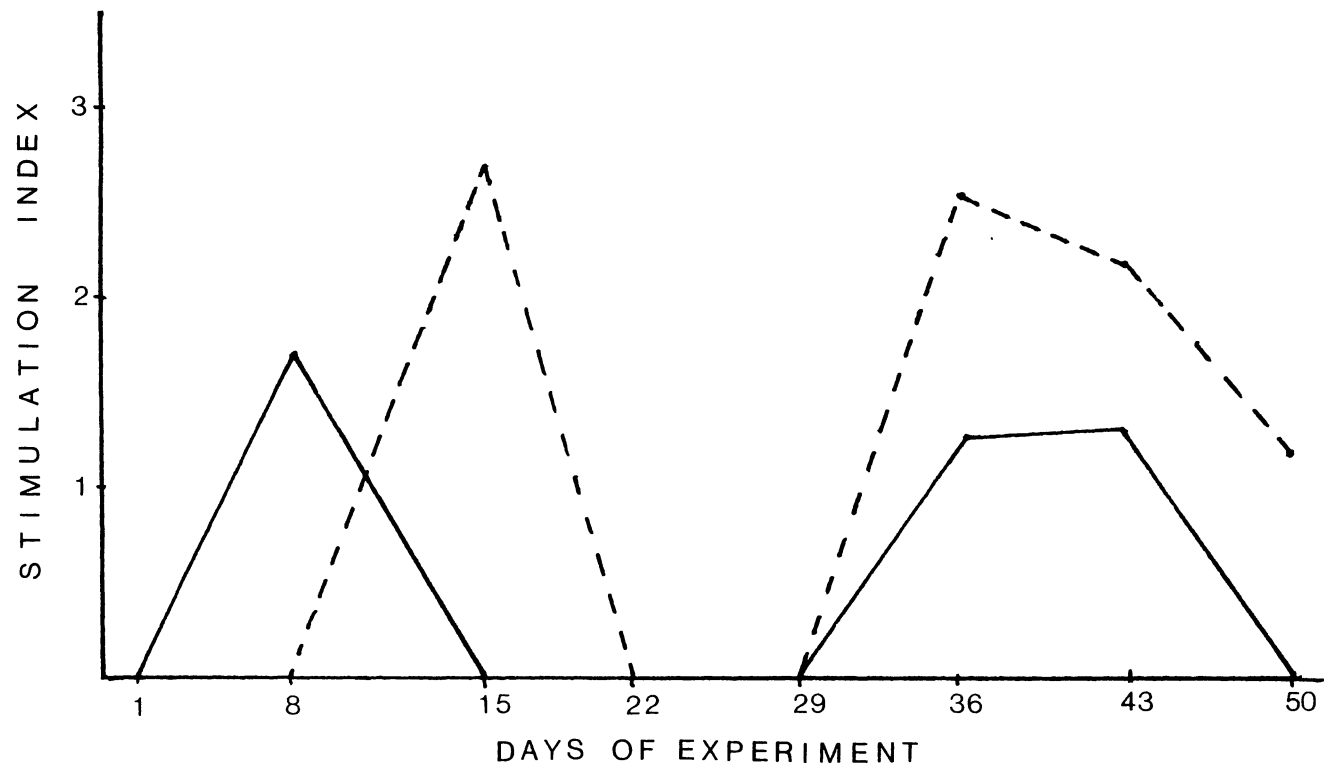


Figure 1. Experiment II: Comparison of Antigen Six Stimulation Indices (SI) of Test Horse B (Solid Line) with the Mean of Control Horses F and G (Dashes). Significant SI are Greater than Two.

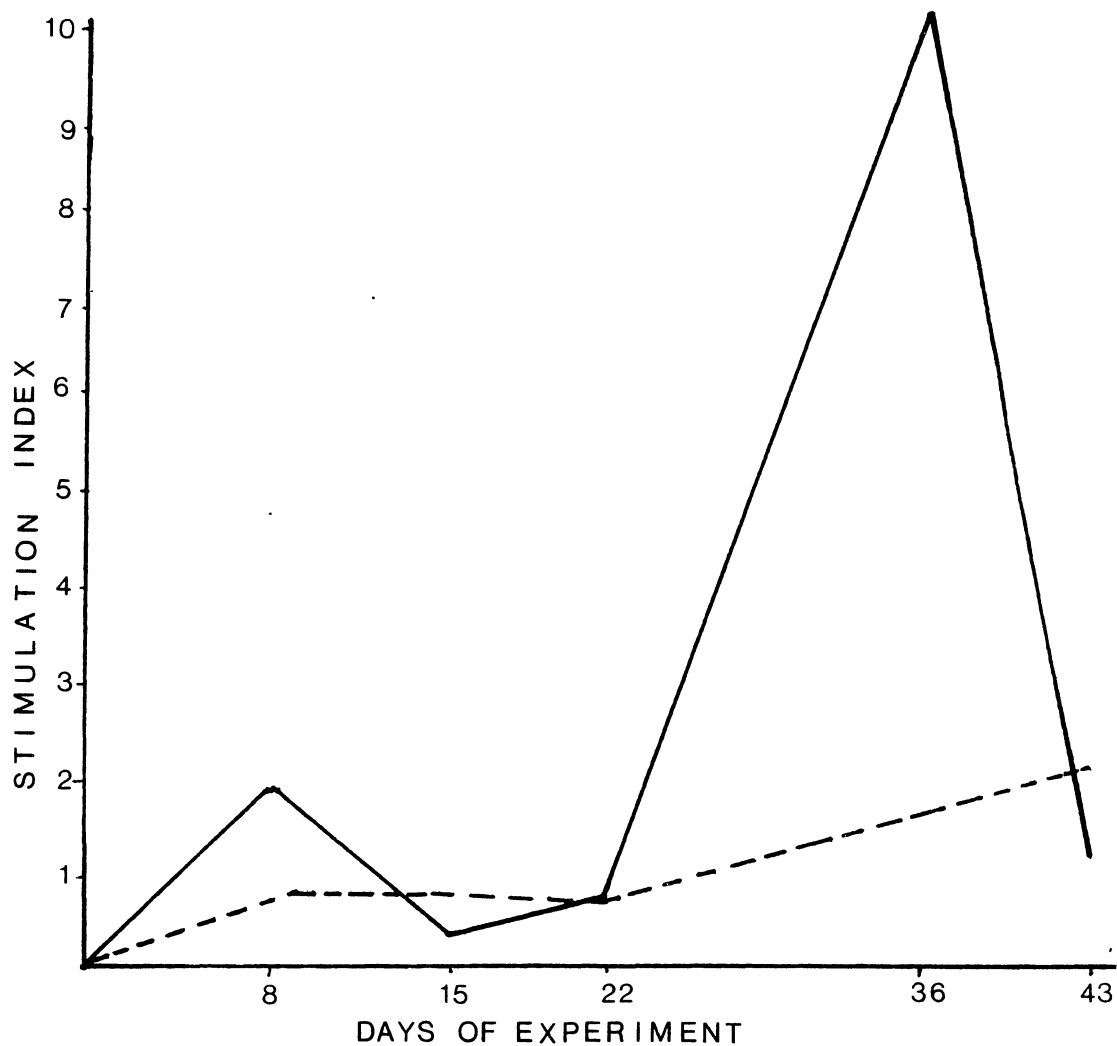


Figure 2. Experiment III: Comparison of Antigen Six Stimulation Indices (SI) of the Mean of Test Horses C, D, and E (Solid Line) with the Mean of Control Horses F and G (Dashes). Significant SI are Greater than Two.

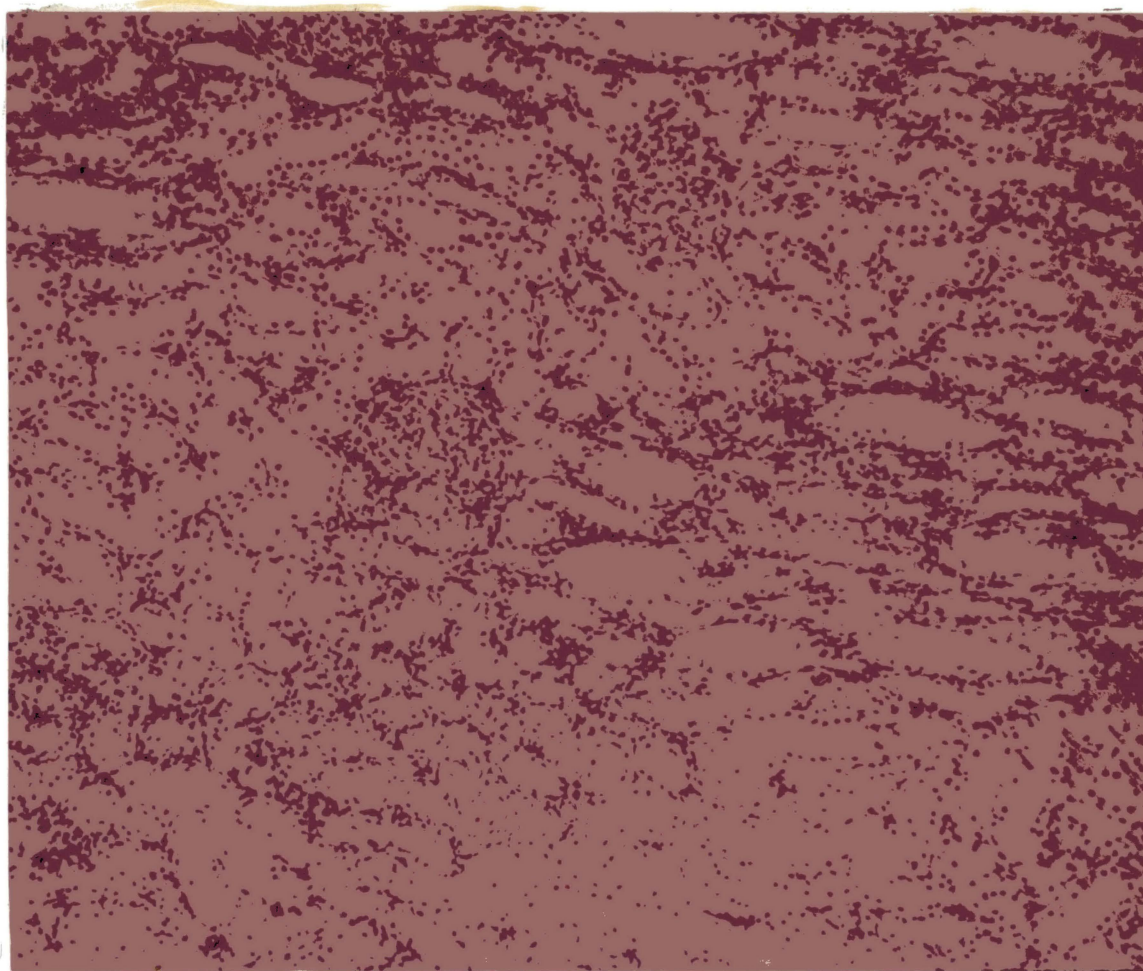


Figure 3. Kidney. Horse B. Mild Glomerular Damage.

probably subclinical. In the spleen, the white pulp was not well formed and there was secondary follicle development which would be consistent with a B cell proliferation and a depletion of T cells. The red pulp was hypercellular and there was a significant amount of hemosiderin deposited in the tissue (Figure 4). Small neutrophilic foci associated with necrotic hepatocytes were scattered throughout the liver (Figure 5). These lesions have been called typhoid nodules and are commonly associated with salmonellosis (99). Some of the lesions were chronic, but many consisted solely of PMN's indicating an acute infection. In addition to kidney, spleen and liver, the postvaccination undiluted antigen skin test site was also histologically evaluated to determine if the reaction was an arthus reaction or delayed-type hypersensitivity. The degree and extent of necrosis of the tissue was greatest around the vessels (Figure 6). The reaction was a combination of inflammation and ischemic necrosis but the cause could not be determined by histopathology.

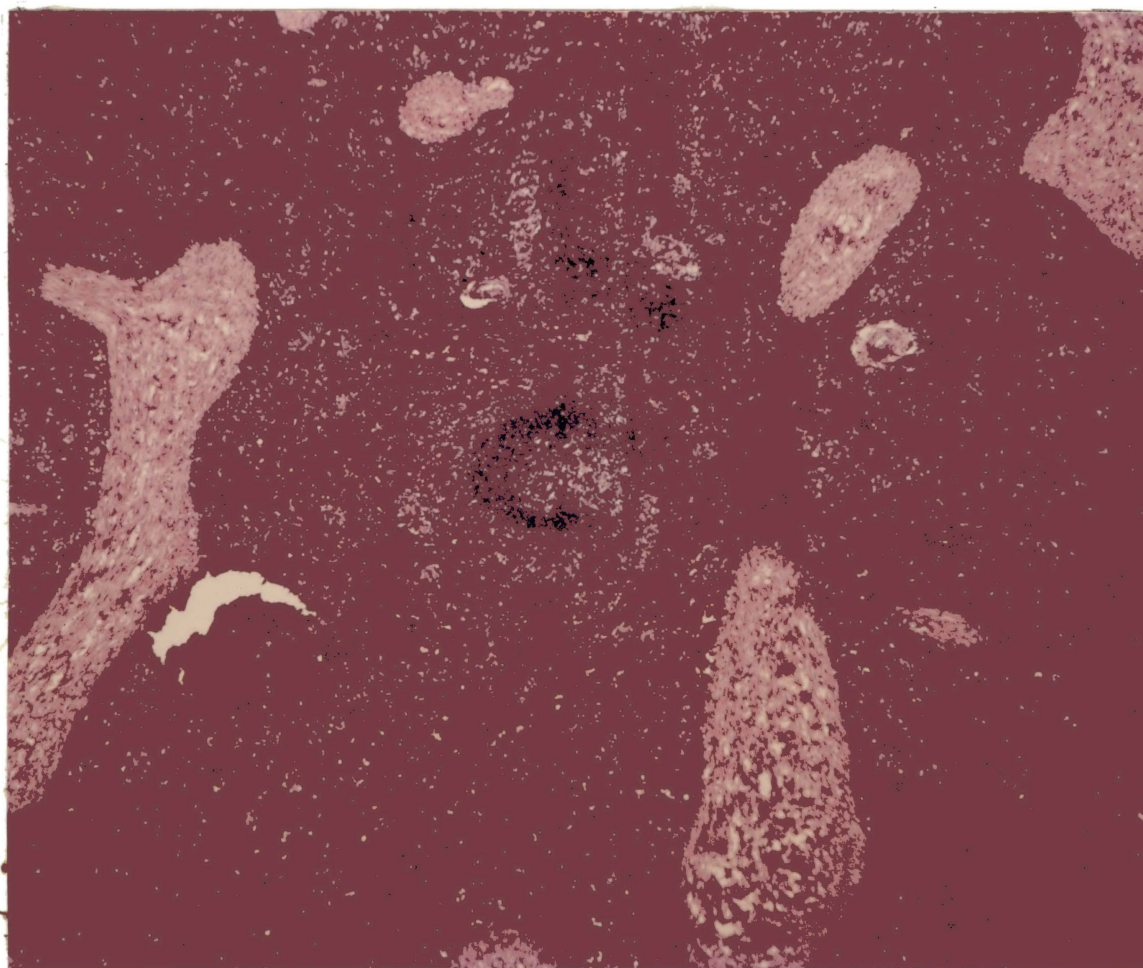


Figure 4. Spleen. Horse B. Secondary Follicle Development and Hemosiderin Deposition.

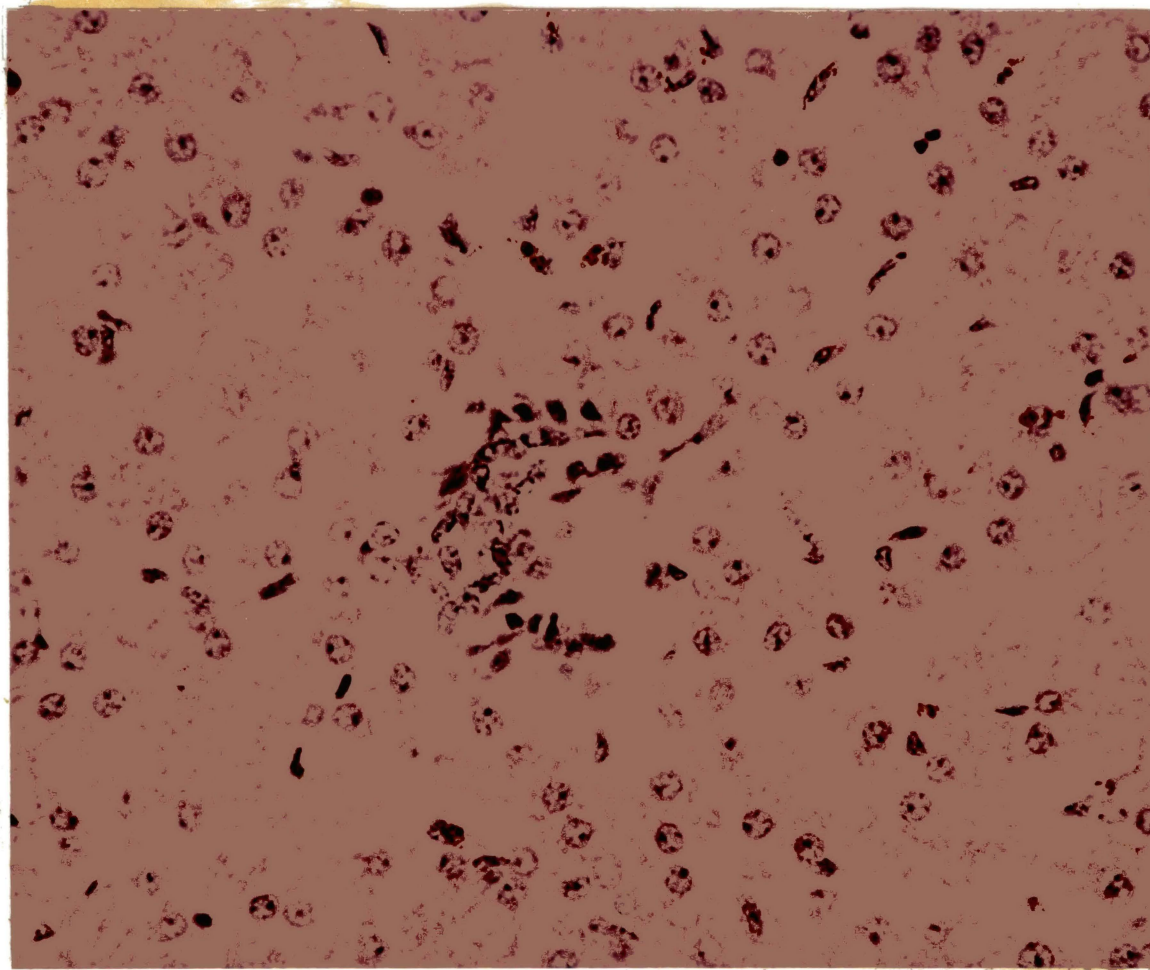


Figure 5. Liver. Horse B. Necrotic Hepatocytes  
Surrounded by Polymorphonuclear  
Leucocytes.

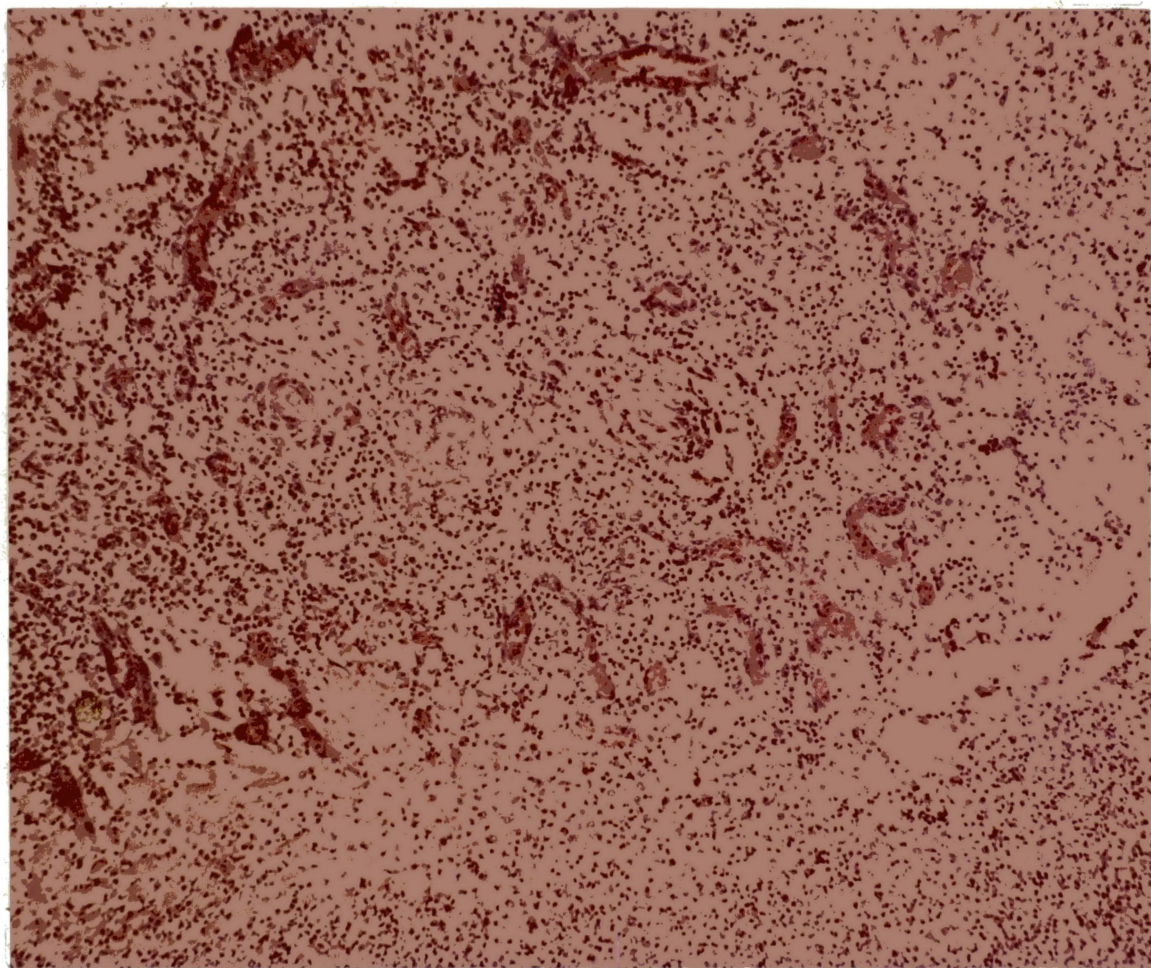


Figure 6. Skin. Test Site. Horse B. Diffuse  
Inflammatory Infiltrate in the  
Dermis.

## CHAPTER V

### DISCUSSION

Salmonella was not isolated from the mesenteric lymph nodes and small intestine of horse A at postmortem, although serotype braenderup was isolated twice from fecal samples during experiment I. There are several explanations for this. One is that the animal was still shedding the organism but in numbers too low to recover culturally. Another is that the horse was no longer infected. The latter is consistent with the clinical signs for this animal; horse A gained weight and developed firmer stools during the experiment.

Serotype agona was cultured from the mesenteric lymph nodes and small intestine of horse B at necropsy but not from two fecal samples taken during experiment II. Salmonella is known to infect and persist in the regional lymph nodes of carrier horses even though fecal culture is negative. Horse B was the most cachectic animal in the study and the isolation of salmonella from this animal was consistent with the clinical appearance of this horse.

The first isolation of serotype agona from horses C, D, and E was made when they were less than six months old, a very susceptible age group. These animals were seen only once a week and feces were collected from the corrals where the horses were individually housed. Although horses C, D, and E had been culturally identified as shedders of serotype agona, the organism was never recovered from fecal samples

during experiment III. It is possible that these animals were still infected and shedding the bacteria in low numbers or carrying the bacteria in the regional lymph nodes. Such inconsistencies in isolation have been noted in the literature. A positive culture can follow a series of negative ones (8). Another possibility is that horses C, D, and E were no longer carriers of serotype agona but had cleared the infection. This is consistent with the clinical appearance of these animals. They gained weight and their stool consistency improved after hyperimmunization.

Although there are many modifications of the fecal enrichment techniques for salmonella isolation described in equine literature, the rate for successful culture is poor. The most consistent isolation of the organism in this study was from horse A where a slightly different cultural technique was used. (Ten grams of feces in 10 ml of PBS were macerated in a Stomaker then used to inoculate selenite and tetrathionate broths which were later used to streak Salmonella-Shigella and brilliant green agar plates.) This technique was used by the Microbiology Laboratory at the College of Veterinary Medicine at Washington State University and gave better results compared to other isolation techniques. A different technique was used in experiments II and III and the cultural results were more variable. Although there was a difference in serotypes between experiments I, II, and III, the cultural technique used in experiment I may have been superior and variability in fecal isolations in later experiments could be due to technique.

All infected horses were skin-tested with undiluted and diluted formalin-killed antigen. The skin test was used to evaluate the cell

mediated immune response (CMI) to antigen. The pre and postvaccination skin test reactions were similar in horse A. The only difference was a postvaccination response to a more dilute concentration of serotype braenderup. A saline control was omitted during skin testing of horse A; however, the omission was not critical to the test since the animal did have some negative reactions to several dilutions. If all the skin test sites had been positive the control would have differentiated reactions due to technique versus reactions to the organism.

The postvaccinational skin test responses of horse B were different from horse A. Horse B had a response to a more dilute antigen and frank necrosis occurred at the site where the undiluted antigen was injected. No definite conclusions could be drawn from histology of the lesion, but the necrosis was worse around the vessels as if they were the primary sites of the reaction. Biopsy of all the skin test sites in horse B would have been valuable because the severity of the reaction to the undiluted antigen made it impossible to determine if a delayed-type hypersensitivity reaction occurred.

The concentrated antigen in experiment III contained live organisms and was inadvertently used to skin test horses C, D, and E. The animals had swellings at all injection sites within four hours but these were gone at 12 hours except for the 1:20 dilution. The animals had no adverse clinical signs and the skin test reactions never appeared infected. However, even with the low numbers of organisms present (0.1 ml of 1:20, 1:40, and 1:80 dilution), use of a live culture may have had a number of effects on these horses. First, the animals were exposed to different antigens, flagellar and cell-wall-associated that are usually changed by formalin or heat-killing. Also, the best

vaccines against intracellular parasites are known to be modified live ones (74, 75). Although there were no clinical signs consistent with bacterial multiplication, it is possible that the skin test may have effected the immune response and changed the results of experiment III.

The prevaccination skin test reactions were similar between horses B, C, D, and E. These animals were infected with serotype agona and were from the same ranch. Horse A did not exhibit as marked a response as horse B to the concentrated antigen dilution. Horse A was infected with serotype braenderup. These results may indicate that different serotypes produce different degrees of response when compared by skin testing. Another possibility for the reduced response of horse A is that the animal was immunosuppressed. This is known to occur in man and animals with chronic infections such as salmonellosis and occurs in horses with chronic diarrhea (83).

Intradermal injection of PHA has been used to evaluate T-cell responses and could have been given concomitantly with other antigen dilutions to estimate CMI. The state of the immune status of infected horses should be known before the animals are used in vaccine studies (102). Even though carriers probably have a reduced immune response, comparison of prevaccination response is low. The comparison may indicate that a response did occur although low compared to normal. Or if there was no CMI demonstrated after vaccination it could be because the animal was too immunosuppressed to respond to any stimulation. The carrier state for salmonellosis is not well understood in man or animals and baseline data may provide insights.

The vaccination schedule used has not been described in the literature for salmonella bacterins in horses. In general, the series

was tolerated by all the horses. However, as the volumes increased and were given into two sites, the animals exhibited some discomfort and became more difficult to handle. Horses A and B demonstrated no reaction to any of the injections. Horses C, D, and E had a marked swelling at the vaccination site given on day 15 of experiment III. One week later a reduced volume was given with no reactions and biweekly injections were resumed. Since high concentrations of agglutinating antibodies can cause such reactions, titers were determined. Titers had increased three-fold from day one to day 15 in horses D and E, and four-fold in horse C to the H antigen preparation of serotype agona. Two-fold increases occurred to the O antigen preparation for horses D and E, while no increase was noted for horse C. Horse E had a two-fold increase in titer between days one and eight, but no reactions had occurred when the animal was vaccinated on day eight. Also, the day one titer of this horse was greater than that on day 15 for horse C and equal to that of horse D, yet no initial reaction to the first injection occurred in horse E. Therefore, the antibody concentration did not appear related to the reaction. Another explanation for the reaction is that there was a problem with vaccination technique. This is consistent with the absence of vaccinal reactions when hyper-immunization was resumed.

In general, equine clinicians do not use titers when evaluating potential cases of salmonellosis because titers are not commonly performed and there is cross reaction between members of the Enterobacteriaceae. However, there are many tests where cross reactions or high background titers occur. These problems are minimized by ignoring low titers and considering large increases (four-fold) as

significant in paired sera. This study demonstrated that although all equine sera had titers, infected horses had much higher antibody levels to the H antigen preparations of the serotype which caused the infection. This finding has clinical as well as epidemiological significance and should be evaluated in greater detail in other infected horses.

Since CMI is considered to be responsible for protection and cure of infections caused by salmonellosis, a test was needed to evaluate the responses produced by hyperimmunization with an autogenous bacterin. The LIF procedure developed by Dorsey was tested once during a preliminary experiment and appeared to work (95). However, there was never migration of the control white blood cells during experiment II. Changes in agarose, serum and white blood cell collection techniques did not help. The cells were viable by the trypan blue dye exclusion test; however, this test measures the integrity of the cell membrane not the ability of the cell to produce a product, divide or to move. This test would show viability even if the cells could not function in the test system. Also, the LIF procedure of Dorsey was developed for use in the bovine. Perhaps the system may require additional modifications when equine blood is used. The LIF test was abandoned and the LST test was used instead. The LST results were also variable, however, some variability is common in this test (94).

The stimulation index (SI) was calculated and used to compare antigen and mitogen responses. In general, if the SI is greater than 2.0 the response is significant (94, 105). Antigen dilution six produced higher SI in all horses although on certain test dates there was not stimulation (SI less than 1.0). Although there was no significant difference in SI between test horse B and control horses

F and G to antigen six (horse B was in poor physical condition and had chronic ongoing visceral salmonellosis), the mean for test horses C, D, and E was greater than the mean for control horses F and G when SI was plotted against time. This indicated that antigen six produced some CMI in these horses. However, the SI produced by the mitogens was much less than expected and in some cases less than the SI produced by the antigens in control horses. One explanation for this is that inappropriate mitogen dilutions were used. No preliminary experiment had been done to determine the best concentration in the test system. Instead, a number of mitogen concentrations were used at the same time in order to speed up the experiment. Another possibility was that the cells were damaged or an intoxicant was in the system. However, this seems less likely since stimulation did occur in test and control horses.

Cell mediated immunity is difficult to evaluate. Although the techniques developed are accurate, they are sensitive to minor variations in technique as well as physiological changes in the test animal. The LST test exhibits variability between different "normal" animals and even for the same animal on different test dates. Such variability has caused some investigators to discount the test. However, the alternative tests (MIF, LIF and skin testing) also have problems with variability. Perhaps these problems will be resolved in the future as research on CMI continues.

Clinically, all horses responded favorably to hyperimmunization. Horse A gained weight and developed a firmer stool; horses C, D, and E grew normally and developed better stool consistency and haircoat. Horse B did not respond as well but had no negative reactions to the

vaccination series even though this animal had chronic ongoing visceral salmonellosis. Hyperimmunization appears to provide clinical improvement in horses infected with serotype agona or braenderup and merits more research as well as serious consideration when attempting to cure a chronically infected carrier.

## CHAPTER VI

### SUMMARY

Humoral and cell mediated immunity as well as clinical response were evaluated in five equine carriers of salmonella after hyperimmunization using an autogenous formalin-killed bacterin.

Hyperimmunization consisted of a series of gradually increasing doses of the bacterin given biweekly for a period of time (six to seven weeks) and represented a novel vaccination scheme for an autogenous salmonella bacterin in the horse. The vaccination schedule was well tolerated by the horses. However, as the volumes increased requiring injection into two sites, the animals exhibited some discomfort and became more difficult to handle.

Sera of control and infected horses were titered using a battery of H and O salmonella antigens of various serotypes of Salmonella enteritidis. These included serotypes: heidelberg, newport, agona and anatum which represented groups: C, A, D, and E, respectively. In general, titers to the H antigens of all the serotypes increased in the vaccinated animals during the experiment. Although all horses had titers, even the controls, infected horses had a significantly higher antibody response to the H antigen preparation of the infecting serotype. Equine clinicians do not use titers when evaluating potential cases of salmonellosis because titers are not commonly performed and there is cross reaction between members of the Enterobacteriaceae.

However, this study demonstrated that infected horses had much higher antibody levels to the H antigen preparations of the infecting serotype of salmonella. This finding has clinical as well as epidemiological significance and should be studied further.

Cell mediated immunity was evaluated using the lymphocyte stimulation test. Although the results were variable, especially the response to the mitogen preparations, significant stimulation was demonstrated to Salmonella enteritidis serotype agona antigen concentration six in three of four infected horses, compared to controls, after the series of vaccinations.

No significant adverse effects occurred during hyperimmunization. In fact, four of the five vaccinated horses showed definite clinical improvement including: weight gain, better stool consistency, normal growth and better coats. The horse which showed no improvement was found to have a chronic active case of visceral salmonellosis at postmortem; yet, this animal had no apparent reactions to the vaccination series.

Hyperimmunization with a killed autogenous bacterin produced positive clinical response with only minimal risk and merits further study as an aid to treatment of horses with salmonellosis.

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## APPENDIX

TABLE XIV. Lymphocyte stimulation data for horse B.

Test	Stimulation Indices*						
	Days After Initial Vaccination						
	8	15	22	29	36	43	50
<b>Ag<sup>+</sup></b>							
1	2.11	-- <sup>⊙</sup>	--	--	--	--	--
2	--	--	--	--	--	--	--
3	--	--	1.02	--	--	--	--
4	--	1.34	1.13	--	1.46	--	--
5	1.35	1.08	1.21	1.06	--	--	--
6	1.69	--	--	NA	1.21	1.25	--
7	NA	--	2.02	NA	NA	1.68	1.06
<b>PWM<sup>+</sup></b>							
1	1.43	1.95	1.37	--	2.10	1.70	--
2	--	1.82	--	--	4.06	4.02	--
3	2.48	1.89	1.45	NA	6.09	3.31	--
<b>PHA<sup>⊙</sup></b>							
1	1.33	--	--	--	--	--	--
2	1.29	--	1.50	--	4.11	1.27	--
3	1.64	--	2.88	--	3.87	1.45	--
<b>CON-A<sup>✓</sup></b>							
1	--	1.00	4.92	--	2.63	1.29	--
2	1.60	--	2.13	--	2.16	--	--
3	1.06	10.58	1.79	--	2.47	--	--

\* Stimulation Index (SI) =

$$\frac{\bar{x} \text{ counts per minute for triplicate test cultures}}{\bar{x} \text{ counts per minute for triplicate control cultures}}$$

<sup>+</sup> Serotype agona antigen.

<sup>+</sup> Pokeweed mitogen.

<sup>⊙</sup> Phytohemagglutinin P.

<sup>✓</sup> Concanavalin A

<sup>⊙</sup> SI less than 1.0.

TABLE XV. Lymphocyte stimulation data for control horse F.

Test	Stimulation Indices*						
	Days of Experiment II						
	8	15	22	29	36	43	50
<b>Ag<sup>+</sup></b>							
1	-- <sup>Ⓢ</sup>	5.41	--	--	1.20	--	--
2	--	2.47	--	--	1.11	--	--
3	--	2.79	--	--	--	--	--
4	--	1.24	--	--	--	1.19	--
5	--	3.44	--	--	--	2.11	--
6	--	2.70	--	NA	--	1.77	--
7	NA	2.23	--	NA	NA	2.07	--
<b>PWM<sup>⊖</sup></b>							
1	1.02	2.19	1.60	--	--	10.31	3.46
2	1.09	9.04	3.93	--	--	14.71	4.17
3	2.22	18.43	7.15	NA	--	11.18	6.88
<b>PHA<sup>⊕</sup></b>							
1	--	2.25	--	--	2.74	--	--
2	1.02	3.98	1.45	--	1.55	11.11	1.81
3	--	4.87	2.16	NA	16.57	13.30	2.82
<b>CON-A<sup>✓</sup></b>							
1	--	2.02	1.29	--	5.01	6.52	--
2	2.34	12.23	1.68	--	6.19	2.58	--
3	1.97	38.82	4.24	NA	3.41	2.82	--

\* Stimulation Index (SI) =

$$\frac{\bar{x} \text{ counts per minute for triplicate test cultures}}{\bar{x} \text{ counts per minute for triplicate control cultures}}$$

<sup>+</sup> Serotype agona antigen.

<sup>⊖</sup> Pokeweed mitogen.

<sup>⊕</sup> Phytohemagglutinin P.

<sup>✓</sup> Concanavalin A.

<sup>Ⓢ</sup> SI less than 1.0.

TABLE XVI. Lymphocyte stimulation data for control horse G.

Test	Stimulation Indices*				
	Days of Experiment II				
	22	29	36	43	50
Ag <sup>+</sup>					
1	1.21	-- <sup>ⓐ</sup>	--	--	--
2	--	--	--	2.33	--
3	1.21	--	--	1.45	--
4	--	1.22	--	1.79	--
5	2.42	1.71	1.30	2.74	--
6	--	--	2.50	2.53	1.16
7	2.19	--	3.76	1.50	2.32
8	--	--	--	2.09	1.09
9	--	--	--	--	5.69
10	--	--	--	4.64	2.80
PWM <sup>Ⓢ</sup>					
1	2.42	--	--	2.74	1.04
2	1.22	--	1.07	5.02	2.12
3	1.74	--	1.33	5.10	3.41
PHA <sup>Ⓢ</sup>					
1	--	--	--	--	--
2	1.22	1.18	2.82	2.30	--
3	--	--	1.29	2.30	--
CON-A <sup>✓</sup>					
1	--	1.94	2.19	3.14	--
2	6.13	1.45	--	--	--
3	--	--	--	1.19	--

\* Stimulation Index (SI) =

$$\frac{\bar{x} \text{ counts per minute for triplicate test cultures}}{\bar{x} \text{ counts per minute for triplicate control cultures}}$$

<sup>+</sup> Serotype agona antigen.

<sup>Ⓢ</sup> Pokeweed mitogen.

<sup>Ⓢ</sup> Phytohemagglutinin P.

<sup>✓</sup> Concanavalin A.

<sup>ⓐ</sup> SI less than 1.0.

TABLE XVIII. Lymphocyte stimulation data for horse C.

Test	Stimulation Indices*				
	Days After Initial Vaccination				
	8	15	22	36	43
Ag <sup>+</sup>					
3	-- <sup>⊖</sup>	--	--	--	1.14
4	3.51	--	--	1.38	1.93
5	1.53	--	--	2.79	1.81
6	4.27	--	--	--	1.13
7	2.33	--	--	--	--
8	1.54	--	--	--	--
9	1.22	--	--	--	--
10	4.85	--	--	--	2.04
PWM <sup>⊕</sup>					
1	1.07	--	--	--	--
2	3.29	--	--	1.25	--
3	1.89	--	--	--	1.59
PHA <sup>⊙</sup>					
1	2.58	--	1.75	1.96	--
2	3.12	--	1.12	1.41	--
3	2.15	--	1.14	--	--
CON-A <sup>✓</sup>					
1	1.55	--	--	--	1.23
2	--	--	--	1.04	1.49
3	3.04	--	--	3.15	--

\* Stimulation Index (SI) =

$$\frac{\bar{x} \text{ counts per minute for triplicate test cultures}}{\bar{x} \text{ counts per minute for triplicate control cultures}}$$

<sup>+</sup> Serotype agona antigen.

<sup>⊕</sup> Pokeweed mitogen.

<sup>⊙</sup> Phytohemmagglutinin P.

<sup>✓</sup> Concanavalin A.

<sup>⊖</sup> SI less than 1.0.

TABLE XVIII. Lymphocyte stimulation data for horse D.

Test	Stimulation Indices*				
	Days After Initial Vaccination				
	8	15	22	36	43
Ag <sup>+</sup>					
3	-- <sup>⊙</sup>	--	1.26	3.72	--
4	--	--	--	--	--
5	--	--	--	2.80	--
6	--	--	--	6.31	--
7	--	--	--	--	--
8	--	1.52	--	1.04	--
9	--	--	--	1.78	--
10	--	--	--	10.10	--
PWM <sup>⊕</sup>					
1	--	--	--	4.38	--
2	--	--	1.39	1.61	1.22
3	--	--	--	2.56	--
PHA <sup>⊙</sup>					
1	--	--	--	3.23	1.15
2	--	--	--	2.72	--
3	--	--	--	2.25	--
CON-A <sup>✓</sup>					
1	--	--	--	--	--
2	--	--	--	1.49	2.11
3	--	--	--	3.02	1.75

\* Stimulation Index (SI) =

$$\frac{\bar{x} \text{ counts per minute for triplicate test cultures}}{\bar{x} \text{ counts per minute for triplicate control cultures}}$$

<sup>+</sup> Serotype agona antigen.

<sup>⊕</sup> Pokeweed mitogen.

<sup>⊙</sup> Phytohemmagglutinin P.

<sup>✓</sup> Concanavalin A.

<sup>⊙</sup> SI less than 1.0.

TABLE XIX. Lymphocyte stimulation data for horse E.

Test	Stimulation Indices*				
	Days After Initial Vaccination				
	8	15	22	36	43
<b>Ag<sup>+</sup></b>					
3	-- <sup>Ⓢ</sup>	--	--	14.27	--
4	--	--	--	25.96	1.94
5	--	--	--	2.02	--
6	1.18	--	--	25.27	1.98
7	1.17	--	--	--	--
8	--	--	--	2.02	3.05
9	1.00	--	--	6.57	2.23
10	1.64	--	--	6.65	--
<b>PWM<sup>Ⓢ</sup></b>					
1	--	--	--	18.84	--
2	2.63	1.27	--	8.43	4.14
3	2.16	8.13	--	1.25	1.76
<b>PHA<sup>Ⓢ</sup></b>					
1	1.36	1.60	--	2.53	5.50
2	1.50	--	1.24	1.21	2.05
3	1.25	2.91	--	3.73	1.26
<b>CON-A<sup>✓</sup></b>					
1	--	2.56	--	--	1.37
2	--	2.00	--	--	--
3	1.59	--	--	--	--

\*Stimulation Index (SI) =

$$\frac{\bar{x} \text{ counts per minute for triplicate test cultures}}{\bar{x} \text{ counts per minute for triplicate control cultures}}$$

<sup>+</sup>Serotype agona antigen.

<sup>Ⓢ</sup>Pokeweed mitogen.

<sup>Ⓢ</sup>Phytohemmagglutinin P.

<sup>✓</sup>Concanavalin A.

<sup>Ⓢ</sup>SI less than 1.0.

TABLE XX. Lymphocyte stimulation data for control horse F.

Test	Stimulation Indices*				
	Days of Experiment III				
	8	15	22	36	43
Ag <sup>+</sup>					
3	-- <sup>Ⓢ</sup>	--	2.41	--	--
4	--	--	--	1.00	1.68
5	--	--	1.65	--	1.09
6	--	--	--	--	4.07
7	--	1.70	2.00	1.02	4.61
8	--	--	--	--	1.22
9	--	1.29	2.62	--	--
10	--	--	2.19	--	1.23
PWM <sup>⊕</sup>					
1	--	--	2.24	--	1.86
2	--	--	4.75	--	2.39
3	--	--	4.47	--	6.52
PHA <sup>⊙</sup>					
1	--	--	3.06	--	3.90
2	--	--	2.31	--	4.16
3	--	--	10.31	--	--
CON-A <sup>✓</sup>					
1	--	--	--	2.17	1.57
2	--	2.86	2.25	--	--
3	--	--	2.57	--	--

\* Stimulation Index (SI) =

$$\frac{\bar{x} \text{ counts per minute for triplicate test cultures}}{\bar{x} \text{ counts per minute for triplicate control cultures}}$$

<sup>+</sup> Serotype agona antigen.

<sup>⊕</sup> Pokeweed mitogen.

<sup>⊙</sup> Phytohemmagglutinin P.

<sup>✓</sup> Concanavalin A.

<sup>Ⓢ</sup> SI less than 1.0.

TABLE XXI. Lymphocyte stimulation data for control horse G.

Test	Stimulation Indices*				
	Days of Experiment III				
	8	15	22	36	43
Ag <sup>+</sup>					
3	-- <sup>⓪</sup>	--	--	--	--
4	2.03	--	--	--	1.81
5	1.35	--	--	4.52	--
6	--	--	--	2.59	--
7	--	--	--	--	--
8	--	--	--	--	--
9	--	--	--	--	--
10	--	--	--	1.21	--
PWM <sup>⊖</sup>					
1	--	--	--	1.34	--
2	1.72	--	--	--	--
3	--	--	1.21	--	--
PHA <sup>⊙</sup>					
1	--	--	--	2.58	--
2	--	--	--	--	--
3	--	--	1.32	--	--
CON-A <sup>✓</sup>					
1	1.38	1.72	1.15	1.97	--
2	--	1.28	--	3.12	--
3	2.13	--	--	--	--

\* Stimulation Index (SI) =

$$\frac{\bar{x} \text{ counts per minute for triplicate test cultures}}{\bar{x} \text{ counts per minute for triplicate control cultures}}$$

<sup>+</sup> Serotype agona antigen.

<sup>⊖</sup> Pokeweed mitogen.

<sup>⊙</sup> Phytohemmagglutinin P.

<sup>✓</sup> Concanavalin A.

<sup>⓪</sup> SI less than 1.0.

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