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By

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ABSTRACT

STUDIES ON THE IMMUNOBIOLOGY AND SERODIAGNOSIS OF *EPERYTHROZOON SUIS* IN SWINE

By

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Swine eperythrozoonosis (EPE), caused by *Eperythrozoon suis* (*Es*), is characterized by acute icteroanemia. Chronic infections are believed to be associatd with impaired growth and reproductive performance although the nature of chronic EPE is poorly understood in part because of the lack of reliable diagnostic techniques for detecting subclinical infections. This study was conducted to evaluate the current EPE serotest, indirect hemagglutination assay (IHA) and develop improved serodiagnostic assays.

A Petri dish-erythrocyte *in vitro* culture was used to maintain *Es*. After intensive screening, the following conditions were found to be optimal for maintenance of erythrocyte attachment, glycolysis and metabolic incorporation of radiolabeled methionine by *Es*: heparin as the anticoagulant, incubation with Eagle's medium under 5 or 10% CO_2, supplementation with inosine and fetal calf serum, and refreshment of medium.

The effects of incubation temperatures and serum absorption with normal pig erythrocytes on IHA, and the comparisons of IHA, ELISA and western blots using Es^+ and Es^- IHA antigens prepared from infected and noninfected blood,

respectively, demonstrated that antibodies detected in IHA were primarily directed at host antigens, not *Es* antigens, suggesting a lack of specificity of IHA for detecting *Es* antibodies.

Antigen preparations were developed by: 1) high speed centrifugation of *Es* dissociated from erythrocytes using EDTA, and *Es* free in plasma; and 2) differential centrifugation (500g, 10,000g and 100,000g) of *Es* dissociated from hemolysates. The second method provided the most *Es* antigens, especially in 100,000g sediments (100K antigen), as determined by western blot analysis. Identical band appearance of *Es* antigens in western blots and autoradiographs of the 100K antigen prepared from radiolabeled *Es* confirmed that the observed antigens were *Es* derived.

ELISA and dot blot procedures were developed using Es^+ and Es^- 100K antigens. In each procedure, CrudeEs-Test and NetEs-Test, based on the Es^+ antigen value only, and the subtraction of the Es^- antigen value from the Es^+ antigen value, respectively, showed high sensitivity with sera obtained from experimentally infected pigs. With sera from naturally exposed pigs, ELISA NetEs-Test was superior to CrudeEs-Test in detecting *Es* antibodies, as determined by comparison with western blot data.

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LIST OF ABBREVIATIONS

A. sp. ----------*Anaplasma* sp.

ABTS ----2,2'-Azino-Bis(3-Ethylbenzthiazoline-6-Sulfonic) Acid

AHDL ----------Animal Health Diagnostic Laboratory

ATP ----------Adenine Triphosphate

B. sp. ----------*Babesia* sp.

BSA ----------Bovine Serum Albumin

CBC ----------Complete Blood Count

CPM ----------Count Per Minute

DB ----------Dot Blot

DNA ----------Deoxyribo Nucleic Acid

E. sp. ----------*Eperythrozoon* sp.

EDTA ----------Ethylenediamine Tetraacetic Acid

ELISA ----------Enzyme-Linked Immuno Sorbent Assay

EM ----------Eagle's Miminum Essential Medium

EMI ----------EM containing Inosine

EPE ----------Swine Eperythrozoonosis

Es ----------*Eperythrozoon suis*

Es^+ ----------*Eperythrozoon suis* positive

Es^- ----------*Eperythrozoon suis* negative

EXP ----------Experiment

FCS ----------Fetal Calf Serum

G-RBC ----------Glutaraldehyde treated Sheep Red Blood Cell

H. sp. ----------*Haemobartonella* sp.

HEPES ----N-2-Hydroxy-Ethylpiperazine-N'-2-Ethanesulfonic Acid

HRP ----------Horse Raddish Peroxidase

IHA ----------Indirect Hemagglutination Assay

IM ----------Intramuscular

IRBC ----------Infected Red Blood Cell

IV ----------Intraveneous

MM ----------Mycoplasma Medium

MW ----------Molecular Weight

N-Ag ----------Negative Antigen

NC ----------Nitrocellulose

OD ----------Optical Density

P. sp. ----------*Plasmodium* sp.

PAGE ----------Polyacrylamide Gel Electrophoresis

PBS ----------Phosphate Buffered Saline

PCR ----------Polymerase Chain Reaction

ProA ----------Protein A

PS ----------Pig Serum

PVP ----------Polyvinyl Pyrrolidone

RBC ----------Red Blood Cell

rEM ----------Reduced Eagle's Minimum Essential Medium

rEMI ----------rEM containing Inosine

RNA ----------Riboxy Nucleic Acid

S-RBC ----------Sensitized Sheep Red Blood Cell

SDS ----------Sodium Dodecyl Sulfate

SPF ----------Specific-Pathogen-Free

TBS ----------Tris Buffered Saline

URBC ----------Uninfected Red Blood Cell

WB ----------Whole Blood

WPI ----------Week Post-Infection

INTRODUCTION

Swine eperythrozoonosis is caused by *Eperythrozoon suis* (*E. suis*), a blood borne rickettsial parasite. The disease was first recognized in the early 1930's in young pigs exhibiting icterus, anemia and depression (Doyle, 1932; Kinsley, 1932). The acute disease is indeed characterized by icteroanemia, however, the majority of infections are subclinical, and recovered animals remain carriers for life.

Prior to the 1970's, the diagnosis of swine eperythrozoonosis was based on herd and individual animal histories describing icteroanemia, the demonstration of *Eperythrozoon* bodies in blood smears (parasitemia) and the complement fixation test (Splitter, 1958). A low level of infection was not detected by these diagnostic techniques and was not recognized to be associated with clinical disease at that time. *E. suis* was considered primarily a pathogen of feeder pigs causing a disease with a low morbidity and high case mortality (Splitter, 1950b). In the mid-1970's, an indirect hemagglutination assay (IHA) was developed (Smith & Rahn, 1975). This assay was aimed at detecting circulating antibodies induced by *E. suis* infection. IHA positive, non-parasitemic animals have been observed routinely and were

considered to be carriers of *E. suis*. A low level, chronic infection is now believed to be associated with impaired growth and reproductive performance although these clinical signs have been widely debated. The disease is thought to be widespread in the midwestern United States.

At present, IHA is the standard diagnostic test for detecting *E. suis* infections. A positive IHA titer indicates an increase in circulating IgM antibodies, and has been correlated with the development of anemia but not with development of detectable parasitemia (Zachary & Smith, 1985). Anemia in eperythrozoonosis has been associated with autoimmunity to altered erythrocyte membranes (Hoffmann et al., 1981; Zachary & Smith, 1985) and IgM cold agglutinin plays an important role in the development of the anemia (Schmidt et al., 1992). These results, plus the likelihood that the antigen used in the IHA test is a crude preparation that contains host erythrocyte antigens, suggest the possibility that the IgM antibodies detected in the IHA may be directed at host erythrocyte antigens as well as *E. suis* antigens, thus potentially reducing the specificity of IHA. Furthermore, Smith (1981) has reported that IHA negative animals can develop parasitemia after splenectomy, indicating that the test may also lack sensitivity with respect to identifying subclinical infections and carrier animals.

Monetary losses due to swine eperythrozoonosis in the United States are unknown and it is often difficult to determine losses in an individual herd because of the lack of

complete understanding of the nature of the disease and definitive diagnostic aids. In the past, inclusion of low levels of relatively inexpensive arsanilic acid in swine diets to control the disease was quite common. However, a change in feed medication regulations in 1986, resulting in a 300-400% increase in medication costs, has heightened producer and veterinarian awareness of swine eperythrozoonosis and related control measures. The response of many producers to the new regulations was to remove arsanilic acid from the diets rather than incur higher medication costs. Subsequently, it appears that there has been an increase in the incidence of swine eperythrozoonosis with the decreased use of arsanilic acid in swine diets. The number of requests for swine eperythrozoonosis serotesting (IHA) and the percentage of positive samples at the Michigan Animal Health Diagnostic Laboratory (AHDL) are depicted in Table 0-1.

Table 0-1. Swine eperythrozoonosis test (indirect hemagglutination assay) performed by the Michigan Animal Health Diagnostic Laboratory during 1983 to 1992.

Year	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992
No. sera*	196	294	327	384	385	385	758	648	181	278
% positive	8.2	19.7	27.2	29.2	25.0	34.8	32.6	36.0	29.3	26.3

* = Number of sera submitted for swine eperythrozoonosis testing.

However, there is considerable controversy over the disease causing role of *E. suis* infection as well as over the reliability of the IHA for detecting *E. suis* infection. A crucial missing component in studying swine eperythrozoonosis under field conditions is the lack of specific and sensitive diagnostic tests.

CHAPTER 1

LITERATURE REVIEW

CLASSIFICATION AND SPECIES OF *EPERYTHROZOON*

According to Bergey's Manual of Systematic Bacteriology, the genus *Eperythrozoon* (Schilling, 1928) is a member of the family, Anaplasmataceae, in the order, Rickettsiales (Kreier & Ristic, 1984). General characteristics of this genus are:

1) the organisms appear as bluish or pinkish violet rings or cocci, 0.4 to 1.5 μm in diameter, in blood smears stained by Romanovsky-type stains;
2) they occur on erythrocytes and free in plasma with about equal frequency;
3) they are obligate parasites of rodents, ruminants, pigs and other vertebrate species.

Eperythrozoon was first reported in mice by Schilling (1928). Since then, *Eperythrozoon* has been reported in many animal species, and Gothe and Kreier (1977) listed 13 species of *Eperythrozoon*. In addition, an *Eperythrozoon* associated with bovine platelets was described in Finland (Tuomi, 1966; Tuomi & von Bonsdorff, 1967). Table 1-1 lists the species of *Eperythrozoon* reported, literature citations, hosts and sites of detection. A number of these listings were probably misclassifications, while others were inadequately described in the literature for meaningful evaluation. For example, the description of *E. noguchii* was based on the appearance of certain objects in photomicrographs published by Noguchi (Weinman, 1944). *E. wenyoni*, *E. teganodes* and *E. tuomii* could be observed subsequently in *E. wenyoni* experimentally inoculated cows (Dr. Y. Nakamura, personal communication).

Table 1-1. Species of *Eperythrozoon* and their characteristics.

Species	Hosts	Sites of detection*	First recorded Author (year)†
E. coccoides	White mouse (*Mus musculus*)	RBC‖, Plasma	Schilling (1928)
E. disper	Dwarf mouse (*Mus minutus*), vole (*Microtus arvalis*)	RBC, Plasma	Bruynoghe & Vassiliadis (1929)¶
E. felis‡	Cat	RBC, Plasma	Clark (1942)
E. leptodactyli	Frog (*Leptodactylus pentadactylus*)	RBC (intracytoplasmic)	Carini (1930)¶, Brumpt (1936)¶
E. mariboi	Flying fox (*Pleropus macrotis epularius*)		Ewers (1971)
E. noguchii	Human		Lwoff & Vaucel (1930)¶
E. ovis	Sheep	RBC, Plasma	Neits et al. (1934)¶
E. parvum	Pig	RBC, Plasma	Splitter (1950a)
E. perekropovi	Pike (*Esox lucius*)		Yakimoff (1931)¶
E. suis§	Pig	RBC, Plasma	Splitter (1950a)
E. wenyoni	Cow	RBC, Plasma	Adler & Ellenbogen (1934)¶
E. teganodes	Cow	Only Plasma	Hoyte (1962)
E. tuomii	Cow	Platelet	Tuomi (1966), Uilenberg (1967)
E. varians	Graybacked deer mouse (*Peromyscus maniculatus gracilis*)		Tyzzer (1942)

* = Sites where *Eperythrozoon* were found.
† = Author(s) who named species (published year)
‡ = *Haemobartonella felis* may be the same species.
§ = The parasite described in Taiwan as *Anaplasma taiwanensis* (Sugimoto, 1935) may be the same species.
‖ = Erythrocyte
¶ = References are in (Weinman, 1944).

Kreier and Ristic (1968) suggested that *E. tuomii* may reflect a life-stage of *E. wenyoni*. Only *E. coccoides*, *E. ovis*, *E. suis*, *E. parvum*, and *E. wenyoni* are accepted species of *Eperythrozoon* in the 1984 edition of Bergey's Manual (Kreier & Ristic, 1984). *Eperythrozoon* like bodies have been reported in other animals including several species of rodents, goats, collared peccaries and recently in llamas (Daddow, 1979; Hannon et al., 1985; Kreier & Ristic, 1968; McLaughlin et al., 1990).

Chronological Review of Swine Eperythrozoonosis

In 1932, inclusions bodies in red blood cells, called *Anaplasma* like bodies, were reported from clinically ill, young pigs (Doyle, 1932; Kinsley, 1932). The main clinical and pathological features of the disease associated with these bodies was icterus and anemia. Presenting clinical signs included fever (40 to 41 °C), depression, weakness, dyspnea, and finally icterus and anemia. A scurvy like condition of the skin in the facial region was also observed (Kinsley, 1932). The disease caused a high case mortality rate (death occurred within 1 to 5 days after clinical signs developed) and low morbidity (<10% in a herd). Microscopic examination of the blood revealed abnormal size and shape of erythrocytes, increased number of segmented neutrophils and the presence of bodies in erythrocytes and free in the plasma (*Eperythrozoon* bodies were thought to be located inside erythrocytes at this time.). These bodies exhibited

considerable variation in morphology including coccoid, bacilliform and ring forms.

Over the next two decades, several authors reported a similar disease characterized by ictero-anemia and the presence of similarly described bodies in red blood cells of feeder pigs 2- to 8-months old and less frequently in other age groups (Campbell, 1945; Quin, 1938; Robb, 1943; Spencer, 1940). The disease was found in pigs in Illinois, Iowa, South Dakota, Missouri (Kinsley & Ray, 1934) and Kansas (Dicke, 1934).

The association between the presence of the bodies in red blood cells and the occurrence of the disease led to naming the disease "anaplasmosis-like disease in swine" (Kinsley, 1939). The acute condition was also known as "ictero-anemia" or "yellow-belly of swine" (Splitter, 1951). Various attempts were made to transmit or to reproduce the disease by injecting the bodies into normal pigs, but experimental inoculations were unsuccessful (Kinsley & Ray, 1934; Robb, 1943; Spencer, 1940), and the etiology of the disease and the nature of the bodies remained unknown. Robb (1943) stated that the bodies in red blood cells were artifacts produced in the process of smearing, staining or drying, based on his finding of the bodies in affected pigs and also in normal pigs. Alternatively, Doyle (1945) suggested that the disease was more likely bartonellosis than anaplasmosis.

After several decades of controversy, the etiology of

the disease was clarified in the 1950's. Splitter and Williamson (1950) found that fever was associated with a high number of the bodies in the blood and that rectal temperatures returned to normal during the period when the bodies disappeared from the peripheral blood. They also described that the bodies were located on the surface of erythrocytes or occasionally free in the plasma, not within erythrocytes as previously reported. Since these features were identical to those of eperythrozoonosis in cattle and sheep, they designated the disease swine eperythrozoonosis. Subsequently, the disease was successfully reproduced by inoculation of *E. suis* infected blood into splenectomized pigs or by splenectomy of carrier pigs (Splitter, 1950b). The resulting symptoms, disease process and pathology were identical to those observed in field cases.

Splitter (1950a) observed 2 types of *Eperythrozoon* bodies in swine, which were different from each other in size and pathogenicity. The larger type (0.8-2.5 μm in diameter) was designated as *E. suis*, the causative agent of ictero-anemia, contained more chromatin and was more pathogenic. The smaller type (0.5 μm in diameter) was designated as *E. parvum*, and considered relatively non-pathogenic. The failure of these *Eperythrozoon* bodies to cross-infect calves, sheep and mice, and the failure of *E. wenyoni* and *E. ovis* to infect splenectomized pigs provided strong evidence that these bodies were a new *Eperythrozoon* species. *E. parvum* was found only in experimentally infected pigs and was not

observed under field condition. The presence of *E. parvum* was also reported in splenectomized pigs in South Africa (Jansen, 1952) and in Great Britain (Jennings & Seamer, 1956; Seamer, 1960). Some of the differentiations of *E. suis* and *E. parvum* summarized by Splitter (Splitter, 1953) are shown in Table 1-2.

Foote et al. (1951) hypothesized that a similar icteroanemia condition in swine was caused by a filterable virus, "the virus of Farley". Because of the successful reproduction of this disease syndrome by injecting the filtrates of whole blood, serum or urine from infected swine without accompanying *Eperythrozoon* bodies, the disease was renamed "virus anemia of swine" and *Eperythrozoon* bodies were considered to be either reactionary bodies or artifacts, or were coincidental with the disease. Interestingly, the virus caused fever and anemia in a splenectomized calf. However, Splitter (1951; 1952; 1953) demonstrated that swine eperythrozoonosis was always accompanied by the appearance of *Eperythrozoon* bodies in blood and stated that swine eperythrozoonosis and "virus anemia of swine" are not identical. Other differences of eperythrozoonosis from "virus anemia of swine", such as no reproductivity using the urine of acutely affected pigs and in splenectomized calves, were reported by Splitter (1951; 1952; 1953). Further evidence of successful reproduction of "virus anemia of swine" and the clinical description of a similar syndrome have not been reported.

Table 1-2. Differentiation of *Eperythrozoon suis* and *E. parvum*.

	E. suis	*E. parvum*
Morphology	Large rings (0.8-1.0 μm) with much chromatin. Discoid forms and very large irregular rings (2-3 μm) may be present.	Small rings (0.5-0.8 μm) with numerous coccoid forms of considerably smaller size. Often found in large numbers on single erythrocytes when organisms are rare in blood.
Pathogenicity	Consistently pathogenic in heavy infections in splenectomized or nonsplenectomized pigs. Etiological agent of acute eperythrozoonosis of swine in the field.	Relatively nonpathogenic. May produce mild to severe anemia in some experimental cases. Not known to produce acute eperythrozoonosis in the field.
Susceptibility to neoarsphenamine	Highly susceptible to doses of 15 mg/kg body weight.	Relatively resistant. Doses of 40-45 mg/kg body weight are effective irregularly.
Interference	*E. suis* displaces *E. parvum* when infection is superimposed.	*E. parvum* is suppressed in initial mixed infections with *E. suis*.
Filterability	Usually retained by the fine diatomaceous earth filters and the Seitz *EK* at negative pressures of 100 mm mercury.	Readily passes the fine diatomaceous earth filters and the Seitz *EK*. Uncomplicated strain may be established by filtration of serum containing *E. suis* and *E. parvum*.

With increasing concern for swine eperythrozoonosis, several review articles and case reports were published in the 1950's and 1960's (Adams et al., 1959; Biberstein et al., 1956; Kingsley & Hibbs, 1968; Preston & Greve, 1965; Stauch, 1951). The disease was reported in 13 states, including California, Georgia, Illinois, Indiana, Iowa, Kansas, Missouri, Nebraska, North Dakota, Ohio, South Dakota, Wisconsin and New York (Biberstein et al., 1956).

The development of a serodiagnostic technique using the indirect hemagglutination assay (Smith & Rahn, 1975) led to the detection of low level of infections without accompanying detectable parasitemia. Since then, swine eperythrozoonosis has been clinically evident in pigs of all ages and chronic infections have been believed to be associated with impaired growth rate and reproductive performance (Henry, 1979). To date, swine eperythrozoonosis has been recognized in North and South America, Africa, Europe and Asia including Korea, Taiwan and Japan.

Morphology and Development

By light microscopy, *Eperythrozoon* exhibits polymorphic forms such as coccoid, discoid, rod, comma, ring or chain. It is often difficult or even impossible to differentiate *Eperythrozoon* bodies from stain deposit, cell fragments or other artifacts in Romanovsky stained preparations. Therefore, it is not clear by light microscopy alone that various shapes of the organism demonstrated are due to

polymorphism or artifact. In earlier studies, Kreier and Ristic (Kreier & Ristic, 1963) failed to observe discoid and ring forms with wet preparations in phase contrast microscopy and indicated that the parasite morphology was different from that observed in dry preparations. Later, coccoid, discoid, ring and chain forms were found by scanning and transmission microscopes (Augsten, 1982; Keeton & Jain, 1973; McKee et al., 1973), and parasite polymorphism in shape was clearly established.

As demonstrated by electron microscopy, *E. suis* is enclosed by a single membrane with a diameter 375 to 500 nm. Internal structures include microtubules beneath the cytoplasmic layer; ribosome-like electron-dense granules and short filaments in the cytoplasma; and vacuoles, 110 nm long and 70 nm wide with no apparent internal structures. No distinct cell organelles nor structures resembling a nucleus were identifiable (Pospischil & Hoffmann, 1982). However, RNA and DNA appears to be present in cytoplasma as small granules and filaments, respectively, as indicated by Tanaka et al. (1965). The presence of RNA and DNA in *E. coccoides* was suggested by Peters and Wigand (1955) and in *Haemobartonella felis* by studies using nucleic acid specific fluorescent dye (acridine orange) (Small & Ristic, 1967).

The various shapes of the organism appear to be closely related with developmental stages. Gulland et al. (1987) classified the shapes of *E. ovis* into 6 forms (spheres, commas, rods, rings, chains and multiple forms) and found a

linear relationship between the forms and the degree of parasitemia. The proportion of sphere and rod forms declined as parasitemia increased, whereas the proportion of multiple forms increased as parasitemia increased. Zachary and Basgall (1985) proposed a developmental cycle for *E. suis*. They hypothesized that *E. suis* exhibits three developmental stages, immature, juvenile and mature forms, which correspond to coccoid, discoid and ring forms, respectively, which were previously observed by light microscopy. The immature form was between 0.2 to 0.5 μm in diameter, indirectly attached to the erythrocyte membrane surface and initially produced no erythrocyte membrane deformation. As the immature form enlarged, intracellular vacuoles developed and the organism became embedded in a deep cup-like invagination on the erythrocyte surface, resulting in significant erythrocyte membrane deformation. The juvenile form developed from the immature form by dorsal-ventral flattening or by developing umbilicated centers, - possibly as a result of rupturing intracellular vacuoles. The mature form had a thin concave central area with numerous small depressions, irregularly thickened outer edges, and an increased diameter. Budding of the small immature forms appeared to be a major mechanism of multiplication and occurred in all developmental stages but was most commonly observed from the thickened edges of juvenile and mature forms. Bud formation has also been described in *E. wenyoni* and *E. ovis* (Ichijo et al., 1982b; Keeton & Jain, 1973). Alternatively, structures indicative

of binary fission were observed in *E. wenyoni* and *Haemobartonella muris* (Keeton & Jain, 1973; Tanaka et al., 1965).

Even though a developmental cycle during the acute parasitemic phase has been postulated, the mechanism of *E. suis* survival/development in carrier animals without having detectable parasitemia is not clearly understood. One potential hypothesis is that multiplication of the organism takes place in bone marrow. *E. ovis* and *Haemobartonella muris* were found on immature erythrocytes (reticulocytes and normochromic erythroblasts) in bone marrow (Ichijo et al., 1982b; Tanaka et al., 1965). In ovine eperythrozoonosis, Ichijo et al. (1982b) observed 35% parasitemia in bone marrow, while the degree of parasitemia in peripheral blood was only 4%, and suggested that bone marrow may be a main site of parasite multiplication. Further investigations are required to assess this hypothesis in swine eperythrozoonosis.

Transmission

Since the discovery of swine eperythrozoonosis, the mechanisms of transmission have been widely debated. For successful experimental transmission with reproduction of the disease, splenectomy of pigs is required (Splitter, 1950b) as attempts at reproducing the disease with intact pigs by injecting parasitemic blood resulted in failure (Kinsley & Ray, 1934; Robb, 1943). Splenectomy is known to induce

clinical disease with various hemoparasites including the genus *Eperythrozoon* (Marmoston, 1935). With splenectomized pigs, *E. suis* can be transmitted via inoculation of infected blood by parenteral routes (IM, IV) and orally (Williams & McCain, 1982). The minimum infective dose appears to be low. In an experiment with *E. ovis*, the minimum infective dose was found to be equivalent to one parasitized erythrocyte (Mason & Statham, 1991).

Because of the seasonal occurrence of the disease, primarily summer and fall, arthropod vectors appear to play an important role in transmission. Hog lice, mosquitoes, stable flies and mange mites have been suggested as potential vectors (Claxton & Kunesh, 1975; Dicke, 1934; Quin, 1938; Robb, 1943; Smith, 1981; Spencer, 1940; Splitter, 1950b; Williams & McCain, 1982). Dicke (1934) reported that the incidence of the disease was lower in herds with an adequate hog lice control program compared to herds with no program.

Experimental transmission by various arthropod vectors has been attempted, but no clear understanding has been achieved. Robb (1943) conducted the first attempt to transmit the disease using hog lice (*Haematopinus suis*). A clinically normal, intact pig was housed with a clinically ill pig that was heavily infested with hog lice. The contact pig exhibited some evidence of depression and fever 2 days after contact, but apparently the clinical signs observed in this pig were more likely due to the sudden heavy infestation of lice rather than *E. suis* infection. One reason for

failure of transmission in this experiment may have been that an intact, non-splenectomized pig was used. The importance of mosquitoes (*Culex pipiens* and *Aedes* sp.) and stable flies (*Stomoxys calcitrans*) in transmission has been suggested by the correlation of the occurrence of blood feeding diptera and *E. suis* infections in swine herds (Prullage, 1989). Subsequently, direct mechanical transmission via mosquitoes (*Aedes aegypti*) and stable flies was demonstrated (Prullage, 1989; Williams & McCain, 1982). Ingestion of the organism by most blood-sucking arthropods, including hog lice, has been observed using a fluorescent antibody assay to detect *E. suis* in the arthropods (Prullage, 1989). Experimental transmission via intramuscular injection of a ground debris of lice which infested parasitemic pigs for 7 days was demonstrated; however, direct transfer of infested lice did not transmit the disease in this study (Dr. R. E. Williams, personal communication; Williams & McCain, 1982). The failure to transmit *E. suis* by direct transfer of infested lice may be partly because the 7 day feeding period may not have been long enough for *E. suis* to completely develop. For example, *Anaplasma*, another rickettsial organism, has been shown to develop slowly in ticks, requiring at least 6 to 9 days before the organisms appeared in the salivary glands for subsequent transmission (Kocan et al., 1992).

Arthropods demonstrated to transmit *Eperythrozoon* species are listed in Table 1-3. Most of the successful

Table 1-3. *Eperythrozoon* and their possible arthropod vectors.

Eperythrozoon	Vector species	References
E. suis	mosquito (*Culex pipiens*)	Williams & McCain (1982)
	stable fly (*Stomoxys calcitrans*)	Prullage (1989)
E. parvum	hog louse (*Haematopinus suis*)	Jansen (1952)
		Seamer (1960)
E. ovis	tick (*Haemaphysalis plumbeum* and *Rhipicephalus bursa*)	Nikolskii & Splipchenko (1969)
	sheep ked (*Melophagus ovinus*)	Avakyan et al. (1973),
		Overås (1969)
	mosquito (*Aedes camptorhynchus*)	Howard (1975)
	mosquito (*Culex annulirostris*)	Daddow (1980)
	stable fly (*Stomoxys calcitrans*)	Overås (1969)
E. coccoides	louse (*Polyplax serrata*)	Eliot (1936),
		Berkenkamp (1988)

transmissions were probably due to mechanical transmission except possibly for transmission of *E. parvum* to splenectomized pigs, which was achieved by transferring hog lice after 15 days of infestation on an infected pig and by oral inoculation of ground hog lice (Jansen, 1952; Seamer, 1960). There are no observations available of *E. suis* development in any arthropod vectors. Strictly speaking, no species of arthropods has been experimentally demonstrated to be a biological vector in *Eperythrozoon* transmission.

Mechanical transmission via needles, ear notching, tail docking and other routine surgical procedures may also play a role in transmission. The occurrence of acute disease has been often observed in conjunction with hog cholera vaccination (Kinsley, 1939; Quin, 1938; Spencer, 1940; Splitter & Williamson, 1950) and with castration (Stauch, 1951). The possibile transmission of *E. suis* via contaminated hog cholera vaccine was rejected because phenolized *E. suis* could not be transmitted (Splitter, 1950b). However, the observation that the disease was not reported following vaccination or other surgical procedures during the winter (Splitter, 1950b) suggested that arthropod vectors were more important in *E. suis* transmission.

In utero transmission may be another mode of transmission. The death of new born pigs (24 to 48 hours of age) from severe anemia with *E. suis* infection has been observed by Berrier and Gouge (1954). Preston and Greve (1965) detected the disease in 4-week-old pigs from a louse

free farm where flies and mosquitoes were not present in significant number and concluded that *in utero* transmission had occured. Claxton and Kunesh (1975) reported that *in utero* transmission was demonstrated by Dr. A. R. Smith (University of Illinois), who observed that new born pigs delivered via hysterotomy from a known *E. suis* carrier sow developed parasitemia one week after birth. These pigs had no contact with the sow or other infected pigs. These findings suggest the possibility of *in utero* transmission; however, detection of *E. suis* in fetal pig blood or antibody production against *E. suis* in fetal pigs has not been experimentally demonstrated.

Clinical Signs and Pathology

Swine eperythrozoonosis can be generally classified into three types (acute, subacute and chronic) based on the time course after infection. The acute type was characterized by a rapid onset of clinical signs followed by death one to three days later (Kinsley & Ray, 1934; Quin, 1938; Robb, 1943; Spencer, 1940). The subacute type lasted 4 to 7 days and was usually followed by recovery (Kinsley & Ray, 1934; Robb, 1943; Spencer, 1940). The chronic type represented the majority of infections, was subclinical and affected animals became carriers for life (Smith, 1981). All three types have been recognized in pigs of all ages; however, Henry (1979) reported that pigs less than 5-days-old are the most likely group within a herd to have clinical signs. Common

clinical signs/syndromes recognized in different age groups included:

1) acute or subacute disease as evident by anemia, mild icterus and weakness in newborn pigs,
2) Chronic disease associated with impaired growth in finishing pigs;
3) acute or subacute disease with the classic icteroanemia in stressed feeder pigs;
4) chronic disease associated with impaired reproductive performance in gilts and sows.

Acute and subacute diseases were generally characterized by icterus and anemia (Kinsley, 1932; Splitter, 1950b; Splitter & Williamson, 1950), in conjunction with many of the other clinical signs. Affected animals exhibited fever (Doyle, 1945; Kinsley, 1932; Smith, 1981), depression, weakness, dyspnea (Doyle, 1932), constipation (Robb, 1943), gait abnormality (Campbell, 1945) and even death (Kinsley & Ray, 1934; Robb, 1943; Spencer, 1940). Purpura, cyanosis and gangrenous necrosis of extremities associated with autoantibody production were also described (Hoffmann et al., 1981; Kinsley & Ray, 1934). Hemoglobinuria, a characteristic of autoimmune hemolytic anemia, has not been observed in swine eperythrozoonosis (Smith, 1977; Splitter, 1950b). There is considerable controversy on this issue in ovine eperythrozoonosis. Ovine eperythrozoonosis has been described as a disease not associated with hemoglobinuria because of the extravascular destruction of erythrocytes

(Blood et al., 1983; Sheriff, 1978; Valli, 1985). However, hemoglobinuria in ovine eperythrozoonosis has been reported (Ichijo et al., 1982a; Inada et al., 1985; Mason et al., 1981; Neitz, 1937; Sonoda et al., 1977) and intravascular hemolysis was observed in the kidneys (Sutton, 1979b). Intravascular hemolysis has not been described in swine eperythrozoonosis. At present, there are not enough data available to evaluate this issue.

Clinical signs of acute disease in sows include anorexia, fever, occasional mammary gland and vulva edema, icterus, reduced milk production and abnormal maternal behavior. Acute episodes at weaning apparently resulted in poor conception at the first return-to-estrus after weaning (Brownback, 1981). The occurrence of acute disease in sows is usually associated with stressful conditions such as movement into the farrowing house or regrouping for breeding. Recovered animals continue with a chronic infection and may exhibit general icterus, no or delayed return to estrus, poor conception, early embryonic death, late-gestation abortion and small litter size. Coincidental respiratory and enteric disease, even death from secondary infections, has been noted in chronically affected sows (Henry, 1979). Zinn et al. (1983) classified sows by their indirect hemagglutination assay (IHA) titer and compared the reproductive performance between sows with IHA titer <1:40 versus ≥1:40, and also between sows with IHA titer <1:80 versus ≥1:80. Litters from sows with IHA titer ≥1:40 or ≥1:80 exhibited significantly

lower birth weights, increased numbers of pigs born dead, lower packed cell volumes and lower hemoglobin values, compared to litters from the sows with IHA titer <1:40 or <1:80. The associations between IHA titer and other reproductive failures, delayed return-to-estrus, early embryonic death or late-gestation abortion, were not observed in this study. However, the ability of IHA in detecting true *E. suis* infections is controversial; thus true associations between *E. suis* infection and specific antibody levels, and various reproductive parameters as investigated in this study must also be questioned.

Blood collected from affected animals showed spontaneous agglutination and elevated sedimentation rate (Doyle, 1932; Hoffmann et al., 1981; Robb, 1943). Analysis of thin smears revealed varied size and shape of erythrocytes and erythrophagocytosis (Doyle, 1932). In addition, hyperglobulinemia and hypoglycemia have been observed (Heinritzi, 1989).

Postmortem examination of acutely affected pigs revealed a general icterus, thin and pale blood, hemorrhages in lung, heart and kidney, enlarged and soft spleen, engorged and yellowish liver (Doyle, 1932; Kinsley, 1932), hydrothorax, hydropericardium, ascites, bile staining of stomach and intestinal contents and mucosa (Robb, 1943), swollen, edematous lymph nodes and pulmonary edema (Quin, 1938; Spencer, 1940). Microscopic examination of spleen and liver revealed hyperplasia of the reticuloendothelial cells,

hemosiderosis and congestion in the spleen, central atrophy of liver lobules, hepatic erythrophagocytosis and hemosiderosis, lymphocyte and macrophage infiltration in liver, and albuminous degeneration of the hepatic cells (Kingsley & Hibbs, 1968; Quin, 1938; Robb, 1943; Spencer, 1940; Splitter, 1950b).

Cold Agglutinin

Production of cold agglutinins to erythrocytes appears an important characteristic in *Eperythrozoon* affected animals. Since discovery of *Eperythrozoon*, spontaneous agglutination of blood taken from animals with acute eperythrozoonosis, especially at cold temperature, has been observed repeatedly. Although it has been postulated that *Eperythrozoon* bodies themselves could bridge erythrocytes and induce hemagglutination (Iralu & Ganong, 1983), anti-erythrocytic antibodies are a more likely cause of this phenomenon (Kreier & Ristic, 1968). Antibodies directed against erythrocytes were demonstrated in *E. coccoides* infected mice and *E. suis* infected pigs by: 1) hemagglutination of noninfected red cells by incubation with immune serum; 2) hemolysis of noninfected red cells by incubation with immune serum and complement; 3) the presence of immunoglobulin on red cells taken from infected animals (direct Coombs test); and 4) the detection of immunoglobulin on noninfected red cells after incubation of noninfected red cells and immune serum (Cox & Calaf-Iturri, 1976; Hoffmann et

al., 1981). Schmidt et al. (1992) isolated a cold agglutinin from *E. suis* infected pigs and demonstrated temperature dependent reactivity. Further biochemical analysis indicated that the isolated cold agglutinin consisted of IgM exclusively.

Detection of cold agglutinins has been reported in *Mycoplasma pneumoniae* infection, mononucleosis (Pruzanski & Shumak, 1977a; Pruzanski & Shumak, 1977b) and many other diseases including hemoparasitic diseases such as haemobartonellosis, anaplasmosis, malaria and babesiosis which are associated with anemia and splenomegaly (Bellamy et al., 1978; Oki & Miura, 1970; Schroeder & Ristic, 1965; Soni & Cox, 1974; Soni & Cox, 1975; Thoongsuwan & Cox, 1973; Zulty & Kociba, 1990). Similar to eperythrozoonosis, the majority of cold agglutinins observed in other diseases are IgM and directed at glycoproteins (I antigen in human) of the erythrocyte membrane and possibly similar antigens located on lymphocytes, monocytes, macrophages and normal tissue cells (Lau & Rosse, 1975; Pruzanski & Shumak, 1977a; Pruzanski & Shumak, 1977b). In haemobartonellosis and eperythrozoonosis, alteration of the erythrocyte surface membrane by neuraminidase or glutaraldehyde treatment was required to induce hemagglutination of noninfected red cells, indicating that the cold agglutinins were directed at cryptic or hidden antigens of the erythrocyte membrane which are exposed to the immune system by parasite infection (Zachary & Smith, 1985; Zulty & Kociba, 1990). Interestingly, *Eperythrozoon* and

Anaplasma infection appear to induce new glycoproteins in erythrocyte membranes. Nordelo and Ysern-Caldentey (1982) observed new glycoproteins in *A. marginale* infected erythrocyte membranes by SDS-PAGE analysis. Goff et al. (1986) observed that the agglutinating ability of erythrocytes via lectins increased with *E. wenyoni* or *A. marginale* infections. Lectins are saccharide binding proteins and it was suggested that a new glycoprotein or glycolipid was synthesized or exposed to the erythrocyte surface by *E. wenyoni* or *A. marginale* infection. Elucidating an association of these new glycoproteins and production of cold agglutinin may provide information on the mechanism of cold agglutinin induction by *Eperythrozoon* or *Anaplasma* infection.

PATHOPHYSIOLOGY

The anemia observed in swine eperythrozoonosis has been classified as an acquired autoimmune hemolytic anemia (Hoffmann et al., 1981). Autoantibodies including cold agglutinins have been suggested to act as opsonins in the process of erythrophagocytosis or to activate complement dependent lysis of red cells (Cox & Calaf-Iturri, 1976; Zachary & Smith, 1985).

Deformation of erythrocyte surface membrane may be a primary initiator of the autoimmunity (cold agglutinin production) induced by *E. suis* infection. Even though direct contact of *E. suis* to erythrocyte membranes was not observed

and *E. suis* and erythrocytes were separated by a distance of 30 nm (Pospischil & Hoffmann, 1982), a thread like structure located between the organisms and erythrocyte membrane appears to be an association tool of the organisms for erythrocytes (Ichijo et al., 1982b). The area of the erythrocyte membrane associated with the parasite has been observed to be more electron dense than non-parasitized areas. Furthermore, membrane deformation persisted after parasites disappeared following tetracycline treatment. These observations suggest an alteration of erythrocyte cytoskelton proteins or an alteration of the relationship of structural proteins with integral proteins of the inner membrane surface (Zachary & Basgall, 1985). The degree of association of *Eperythrozoon* to the erythrocyte membrane may explain variations in the degree of pathogenicity with respect to anemia induction. For example, it was observed that *E. wenyoni* which generally induces no or slight anemia in cows caused only slight depression of erythrocyte membranes and did not cause electron density alteration of the erythrocyte membrane (Keeton & Jain, 1973).

Cyanosis of the skin, especially in the extremities, has been occasionally observed in affected pigs, presumably the result of cold agglutinin (autoantibody) production. The cold agglutinins induced by *E. suis* infection cause agglutination, restricted circulation and necrosis in the skin because the skin temperature is lower than the core body temperature (Heinritzi et al., 1990a).

Little is known about the pathophysiology of impaired growth resulting in "delayed marketing syndrome". Eperythrozoonosis is considered to be associated with stress, especially social stress, as is seen in certain protozoal diseases in which the host parasite relationship is guided by premunition. However, Nicholls et al. (1989) failed to show stress induced by feed or exercise restriction affected the growth rate of *E. ovis* infected sheep.

Hypoglycemia, a common feature in acutely affected pigs, may partially explain the impaired growth in chronically infected finishing pigs and also the weakness observed in acutely affected baby pigs. Investigation of metabolic changes in experimentally infected, splenectomized animals found decreased concentration of serum glucose, increased concentration of blood pyruvate and lactate, and decreased blood pH (Heinritzi et al., 1990b; Heinritzi et al., 1990c), indicating activation of glycolysis. A decreased blood glucose concentration (up to 25% at 23 days post infection) was also observed in experimentally infected, intact pigs (Heinritzi et al., 1990b). Similar observations were reported in *E. ovis* infected sheep (Ilemobade & Blotkamp, 1978b; Sutton, 1976; Sutton, 1977). These observations suggested that *Eperythrozoon* themselves have a high glycolytic activity or *Eperythrozoon* may have a profound effect on erythrocyte glycolytic activity, despite their semi-extracellular location. High glucose utilization, either as a result of parasite metabolism or enhanced

erythrocyte metabolism caused by the parasite, may cause generalized hypoglycemia (Wensing et al., 1974) and affect growth rates. This seems especially important in piglets which are known to have a precarious energy balance at birth (English & Smith, 1975) and may partly explain the mechanisms of the perinatal problems (stillbirth, weak pigs) observed in swine eperythrozoonosis.

Impaired erythrocyte function could be postulated as a mechanism for impaired growth. Lactate production as a result of massive glycolysis leads to metabolic acidosis, which is usually combined with respiratory acidosis caused by impaired pulmonary gas exchange (Heinritzi et al., 1990b). Low blood pH leads to shifts in the oxyhemoglobin curve and, subsequently, the delivery of oxygen to peripheral tissue may be impaired. In ovine eperythrozoonosis, reduced glutathione levels were observed in *E. ovis* infected sheep (Sutton, 1979a). Glutathione is an essential component of erythrocytes for removing peroxide which is produced as methemoglobin is formed. Therefore, the effect of *E. ovis* on glutathione concentration might interfere to some extent with erythrocyte function.

Hypoglycemia may also be related with some aspects of impaired reproductive performance. For example, it has been reported that insulin regulates ovulation rate (Matamoros et al., 1991). Insulin administration affects follicular dynamics by reducing follicular atresia and increasing the number of potential ovulatory follicles. It could be

expected that hypoglycemia induced by *E. suis* infection may lead to low endogenous insulin levels. Perhaps, the impaired reproduction observed in swine eperythrozoonosis, such as delayed puberty in gilts and delayed return-to-estrus in sows, may be explained by low insulin levels due to chronic hypoglycemia. At present, insulin levels in *E. suis* infected sows nor the pathophysiology of swine eperythrozoonosis in reproducing sows has not been studied although such studies appear to be warranted.

Host Immunity

As observed with other hemoparasitic diseases such as malaria and haemobartonellosis (Maede, 1978; Weiss et al., 1986), the reticuloendothelial system plays an important role in eliminating *Eperythrozoon* species from the circulation. Splenomegaly is an important characteristic of *Eperythrozoon* infections and *E. coccoides* has been called the "Spleen Weight Increasing Factor" (Stansly, 1965). Splenectomy induces severe parasitemia both in primary infected animals and in carrier state animals (Marmoston, 1935; Rouse & Johnson, 1966; Splitter, 1950b). Therefore, the spleen plays a critical role in the sequestration and elimination of the *Eperythrozoon* organisms (Baker et al., 1971).

Detailed studies of splenic function have been conducted using electron microscopy in swine and ovine eperythrozoonosis. Reported observations included: 1) cordal reticular cells and macrophages phagocytized both parasitized

and non-parasitized red cells; 2) pseudopodia extending from the reticular cells and macrophages appeared to engulf and remove *Eperythrozoon* organisms without destroying red cells; 3) parasitized red cells did not pass through basement membrane fenestrations and were likely to remain in the cord, thus facilitating phagocytosis in the cord (Hung & Lloyd, 1989; Pospischil & Hoffmann, 1982). Removal of organisms from erythrocytes by extended pseudopodia of reticular cells and trapping of organisms by pseudopodia at the basement membrane fenestrations were also reported in feline haemobartonellosis (Maede, 1979). Maede (1978) observed that *H. felis* infected red cells labeled with ^{51}Cr were highly sequestered in the spleen of intact animals, and in the liver, lung and bone marrow of splenectomized animals. These findings indicated that the spleen is a major organ for eliminating the organism from circulation, and liver, lung and bone marrow might also contribute to some extent (Baker et al., 1971; Maede, 1979).

Mononuclear phagocytic activity has been noted to be markedly increased during infection with *Eperythrozoon* and *Haemobartonella* species as measured by carbon clearance from blood (Elko & Cantrell, 1968; Gledhill et al., 1965). Mice inoculated with *E. coccoides* or rats inoculated with *H. muris* and subsequently challenged with mouse hepatitis virus (Gledhill & Dick, 1955), lactate dehydrogenase virus (Riley, 1964; Riley et al., 1964), and lymphocytic choriomeningitis virus (Seamer et al., 1961) exhibited more severe clinical

signs and lesions than animals inoculated with virus alone. Baker et al. (1971) suggested that rapid replication of macrophages and increased phagocytosis due to hemotropic parasitic infection made it easier for viruses to enter and replicate in cells.

Interferon responses in the host may contribute to these observations. *E. coccoides* infection suppressed further interferon production in mice in response to viral infection (Glasgow et al., 1974; Glasgow et al., 1971). Conversely, *E. coccoides* infection by itself induced low levels of interferon production (Suntharasamai & Rytel, 1973). This alternate finding may partially explain observations that *E. coccoides* infected mice resisted superinfection with *Plasmodium berghei* (Peters, 1965), *P. chabaudi* (Ott et al., 1967; Voller & Bidwell, 1968) and *P. vinckei* (Cox, 1966), possibly due to the antiparasitic effect of interferon.

The importance of protective antibodies in host defense mechanisms in swine eperythrozoonosis is not clearly understood although it has been suggested by studies of murine and ovine eperythrozoonosis. Observations which support the presence of protective antibodies include: 1) infected animals were resistant to reinfection; 2) clearance of parasitemia was correlated with the appearance of circulating antibodies; 3) transfer of immune serum to parasitemic animals resulted in the clearance of organisms; and 4) inoculation of *Eperythrozoon* bodies after incubation with immune serum (neutralization) resulted in a prolonged

prepatent period and reduction of parasitemia (Glasgow et al., 1974; Hung & Lloyd, 1985; Hyde et al., 1972; Thurston, 1955). However, Hung and Lloyd (1985) failed to demonstrate an opsonin effect of antibodies in an *in vitro* erythrophagocytosis test with *E. ovis* infected erythrocytes.

Although infected animals become resistant to reinfection, recurring parasitemia after the initial clearance of circulating parasites has been frequently observed (Hung & Lloyd, 1985; Thurston, 1955). Recurring parasitemia has been observed in animals infected with hemoparasites such as *Babesia* and *Eperythrozoon*, and was proposed to be associated with new antigenic variants (Hung & Lloyd, 1985; Rogers, 1974; Thoongsuwan & Cox, 1973). However, detailed studies on antigenic variants of *Eperythrozoon* species have not been reported.

Passive immunity via colostrum was observed in sheep with *E. ovis* and as expected, transplacental transfer of passive antibodies could not be demonstrated in sheep (Daddow, 1982).

A study of lymphocyte function in eperythrozoonosis was conducted by Zachary and Smith (1985). Using a lymphocyte blastogenesis assay, they found polyclonal T cell suppression and polyclonal B cell activation during *E. suis* infection. These findings may explain the often reported increased susceptibility of *Eperythrozoon* infected pigs to other infectious diseases.

Induction of parasitemia using immunosuppressive agents

is a controversial issue. Immunosuppression after administration of cortisone was generally ineffective for inducing detectable parasitemia as observed in *H. muris* (Laskowski et al., 1954; Scheff et al., 1956) and *E. coccoides* infected animals (Thurston, 1955). However, some modification of infections by corticosteroids has been reported. Daddow (1977) used betamethasone in *E. ovis* infected sheep to hasten the development of parasitemia and Martin et al. (Martin et al., 1988) found that dexamethasone treatment enhanced the severity of clinical disease in sheep. Antilymphocyte serum and cyclophosphamide were ineffective for inducing parasitemia of *H. muris* and *E. coccoides* (Baker et al., 1971). Conversely, induction of parasitemia in latently infected animals using anti-splenic agents was successful. Successful induction of parasitemia has been reported with: 1) anti-rat spleen serum (Pomerat et al., 1947; Thomas et al., 1949); 2) ethylpalmitate, which depresses reticuloendothelial activity (Finch et al., 1968); and 3) polonium (^{210}Po), which selectively injures the spleen (Scott & Stannard, 1954).

In summary, splenic function appears to be the core defense mechanism against *E. suis* infection. Activation of the reticuloendothelial system appears to be a major component of eliminating *E. suis* from the circulation. However, the role of humoral immunity in *E. suis* elimination is not well understood, although protective antibodies appear to be produced following infection.

Diagnosis

Prior to 1970, the diagnosis of swine eperythrozoonosis was based on herd and individual animal histories describing ictero-anemia, the demonstration of *Eperythrozoon* bodies in blood smears and the complement fixation serotest (Splitter, 1958). Detection of the organism provides definite diagnosis; however, the majority of *E. suis* infections are subclinical and parasitemia can be detected only in acutely affected animals. Moreover, artifacts in blood smears are often difficult to distinguish from *E. suis* bodies in many cases (Henry, 1979). A low level, chronic infection could not be detected by these classical techniques.

In the mid 1970's, Smith and Rahn (1975) developed an indirect hemagglutination assay (IHA). This assay was aimed at detecting antibodies induced by *E. suis* infection. IHA positive, non-parasitemic pigs have been observed routinely and are considered to be carriers of *E. suis*. The IHA test involves glutaraldehyde attachment of a partially purified *Eperythrozoon* antigen, prepared from the plasma of *E. suis* infected blood, to sheep erythrocytes. Serial serum dilutions are reacted with the treated sheep erythrocytes to determine the serum titer. A positive IHA titer, associated with IgM antibodies to *E. suis*, is correlated with anemia but not with detectable parasitemia, and indicates either a recent or persistent/chronic infection (Zachary & Smith, 1985).

The IHA test is widely used for diagnosis although

clinical interpretations are often confusing. The time course of IHA titer development and decline is poorly understood. As the test measures IgM antibodies, a transient titer rise would be expected from 2-6 weeks following exposure. Experimentally, this transient rise has been observed. However, persisting high titers are frequently observed in naturally infected pigs as well as splenectomized pigs (Baljer et al., 1989). These results, along with the facts that the antigen used in IHA test is a crude preparation that may include host antigens, and that *E. suis* infection induces autoantibody production (Hoffmann et al., 1981) suggests the possibility that IgM antibodies measured in the IHA test may be directed at host erythrocyte antigens, thus potentially reducing the test's specificity. Furthermore, Smith (1981) reported that infected animals which are serologically negative by the IHA test can be carriers because they developed a parasitemia after splenectomy, thus indicating that the test may lack sensitivity with respect to identifying carrier pigs.

Various other serological techniques have been proposed for *Eperythrozoon* spp. antibody detection. Kreier and Ristic (1963) developed an indirect fluorescent test using intact *Eperythrozoon* bodies as the antigen for ovine and bovine eperythrozoonosis. Subsequently, antibodies against *Eperythrozoon* bodies were detected by indirect fluorescent tests in mice (Baker et al., 1971) and sheep (Ilemobade & Blotkamp, 1978a; Nicholls & Veale, 1986). In addition,

complement-fixation tests for murine (Wigand, 1956), ovine (Daddow, 1977) and swine eperythrozoonosis (Splitter, 1958), an indirect hemagglutination assay for bovine eperythrozoonosis (Finerty et al., 1969), and enzyme linked immunosorbent assays (ELISA) for ovine (Lang et al., 1987), bovine (Kawazu et al., 1990) and swine eperythrozoonosis (Hsu et al., 1992; Schuller et al., 1990) have been developed using partially purified antigens prepared from erythrocyte lysates. Considering autoantibody production in *Eperythrozoon* infection, a major concern with these immunoassays is the inclusion of erythrocyte antigens in the antigen preparations. Lang et al. (1986) described the successful separation of *E. ovis* bodies from hemolysates. Following this method, Hall et al. (1988) failed to completely separate *E. suis* bodies from erythrocyte antigens by lysing erythrocytes. Alternatively, they used di-sodium ethylenediamine tetraacetic acid (EDTA) to release the organisms from the erythrocytes. They separated the free organisms from the erythrocytes by low speed centrifugation. The organisms were then concentrated by high speed centrifugation yielding a high concentration of *E. suis* bodies.

Recently, *E. suis* DNA detection techniques have been developed using whole organism DNA hybridization (Oberst et al., 1990b) and recombinant DNA hybridization (Oberst et al., 1990a). These techniques detected *E. suis* specific DNA from the high salt lysate of whole blood samples, and apparently

offer high sensitivity and specificity for detecting *E. suis* infection. These techniques could potentially lead to the development of a more powerful diagnostic technique using the DNA polymerase chain reaction (PCR), in which very low level of infection could be detected by amplification of *E. suis* specific DNA.

Treatment and Control

Laboratory and field studies have demonstrated the beneficial effects of treatment with tetracyclines and/or arsenicals (Henry, 1979; Rosenkrans et al., 1984; Splitter, 1950c; Splitter & Castro, 1957). Individual drugs tested for activity against *Eperythrozoon* species are listed by Gothe and Kreier (1977). Table 1-4 shows drugs tested for activity against swine eperythrozoonosis. Some of these medications have been shown to reduce the clinical signs of affected pigs; however, no drug has been shown to completely clear infection. Although no drug has been approved for the treatment and control of swine eperythrozoonosis at present in the United States, inclusion of low levels of arsanilic acid in swine diets appeared to effectively control the disease (Smith, 1981).

Table 1-4. A list of drugs tested for activity against *Eperythrozoon suis*.

Drug	Dose	Action	Reference
Arsanilic acid	90 g/ton in feed	Prophylaxis	(Smith, 1981)
Chlortetracycline	45 g/ton in feed	Prophylaxis	(Anthony et al., 1962)
Chlortetracycline	200 mg/gal in water	Prophylaxis	(Anthony et al., 1962)
Neoarsphenamine	45 mg/kg IV	Cleared parasites; relapses occured	(Splitter, 1950c)
Oxytetracycline	3 mg/lb IM	Cleared parasites; relapses occured	(Splitter & Castro, 1957)
Sodium cacodylate	0.3 mg/lb IV	No effect	(Splitter, 1950a)
Tetracycline	3 mg/lb IM	Cleared parasites; relapses occured	(Splitter & Castro, 1957)

CHAPTER 2

IN VITRO CULTIVATION OF *EPERYTHROZOON SUIS*

Abstract

In vitro culture of *Eperythrozoon suis* was attempted using a Petri dish-erythrocyte culture system. In preliminary experiments, the following conditions were found to be optimal for maintaining *E. suis* attachment to erythrocytes in cultutre: anticoagulation with heparin or citrate solution, incubation with 5 or 10% CO_2 at 37°C, and incubation with reduced or non-reduced Eagle's minimum essential medium.

Using heparin, a CO_2 incubator and reduced Eagle's medium (rEM), *E. suis* metabolic activity was evaluated by measuring glucose consumption, and lactate and pyruvate production. Glucose consumption and lactate production were apparent while pyruvate production was not detected. Erythrocyte integrity was improved by the addition of inosine, an energy source of swine erythrocytes, although no effect was observed on maintenance of *E. suis* attachment to erythrocytes or the rate of glucose consumption. To determine whether the observed glucose consumption in culture was due to *E. suis* glycolytic activity or enhanced erythrocyte glycolytic activity, the effect of *E. suis* killing by EDTA addition to medium was evaluated using rEM containing inosine (rEMI). Glucose consumption decreased proportionally with the decline in the percentage of parasitized erythrocytes induced by EDTA, indicating glucose consumption was due to *E. suis*. In a subsequent experiment,

the effect of different types of serum (pig or fetal calf serum) and different gaseous environments (5% CO_2 incubator or candle jar) were evaluated using rEMI. Glucose consumption by *E. suis* was significantly increased by the addition of fetal calf serum; however, no difference in the maintenance of *E. suis* attachment to erythrocytes and in *E. suis* glycolytic activity was observed with a 5% CO_2 incubator and candle jar. Finally, the effect of medium refreshment (rEMI containing fetal calf serum) was evaluated. Maintenance of *E. suis* parasitism on erythrocytes and *E. suis* glycolytic activity were significantly improved by frequent medium refreshment. The developed culture system could be used for metabolic radiolabeling of *E. suis* for further protein/antigen analysis.

Introduction

Swine eperythrozoonosis is caused by the rickettsial organism, *Eperythrozoon suis*. The disease is characterized by acute ictero-anemia and is associated with impaired weight gains and reproductive performance (Henry, 1979). Swine eperythrozoonosis has been reported world wide including North and South America, Africa, Europe and Asia (Henry, 1979; Hsu et al., 1990; Sisk et al., 1980; Smith, 1977; Smith, 1981).

Swine eperythrozoonosis is poorly understood in part because of the lack of an *E. suis in vitro* culture system. Organism propagation is typically done by inoculating splenectomized pigs with infected whole blood and then collecting blood from the inoculated pigs as parasitemia develops. The whole blood is used immediately or frozen for future use. The lack of an *in vitro* culture system is a critical barrier preventing systematic serological, immunological and molecular-biological studies of *E. suis*. In comparison, a successful *in vitro* culture system established for *Plasmodium falciparum* (Trager & Jensen, 1976), has contributed significantly to understanding the disease by facilitating studies on antiparasitic compounds (Geary, 1983), and parasite ultrastructure, development, biochemistry, immunology and serology (Jensen, 1983).

The purpose of this study was to develop an *in vitro* culture system for *E. suis*. *In vitro* culture could provide a

uniform method for propagating *E. suis* in a controlled environment, decrease the need for animal experimentation in *E. suis* investigations and facilitate radiolabeling techniques for antigen analysis.

Materials and Methods

Experimental Infections

Organism. E. suis was initially isolated from a parasitemic, febrile boar housed at the Swine Research Center, Michigan State University, and has been maintained by passage through splenectomized pigs. Infected blood also was cryopreserved by adding 6.7% and 10% (v/v) of 40% polyvinyl pyrrolidone (PVP) and glycerol, respectively, and then freezing in liquid nitrogen.

Animals and inoculation of E. suis. Crossbred pigs were 6- to 13-weeks-old at the time of inoculation. All animals were splenectomized using standard surgical techniques. Ten milliliters of frozen, infected blood was thawed in a 37 °C water bath and inoculated intravenously and/or intramuscularly into the splenectomized pigs. Clinical signs and rectal temperature were assessed daily to monitor the development of clinical disease. When the rectal temperature exceeded 40 °C, blood was collected from the jugular vein in a vacutainer tube (Beckton Dickinson Vacutainer Systems, Rutherford, NJ) containing ethylenediamine tetraacetic acid (EDTA) and examined for parasitemia by Wright's stained thin smear. At the height of parasitemia (>75% parasitemia), blood was collected from the anterior vena cava with either sodium heparin (30 U/ml), 0.15% EDTA, 0.38% sodium citrate or

glass beads. Blood collected with glass beads was gently agitated until fibrin formation was observed on the beads. At the same time, blood was collected from noninfected pigs which were housed in a separate building.

When the febrile phase lasted for more than 3 days or if the animals exhibited severe depression, intramuscular treatment with oxytetracycline (20 mg/kg body weight; Liquamycin LA 200, Pfizer, New York, NY) was initiated to avoid mortality.

CULTURE

Media. Several media were used and are described as follows:

1) RPMI 1640 (pH 7.4; GIBCO Laboratories, Grand Island, NY) was supplemented with 25 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.2% $NaHCO_3$ and 1% penicillin-streptomycin solution (Sigma Chemical Co., St. Louis, MO).

2) Mycoplasma medium (MM; pH 7.8) was prepared with PPLO broth (1.5% w/v; Difco), 2.5% yeast extract, 0.1% glucose, 1% penicillin G (1,000 U/ml), 0.2% Thallium acetate and 0.002% phenol red.

3) Eagle's minimum essential medium (EM; pH 7.3) was prepared from powder containing Eagle's medium supplements, non-essential amino acids, Earle's salts and L-glutamine (Sigma Chemical Co.) supplemented with 25 mM HEPES, 0.1%

glucose and 0.2% $NaHCO_3$, in 1100 ml of distilled water. Reduced EM (rEM) was prepared by further addition of 0.2 mM β-mercaptoethanol, 2.0 mM sodium pyruvate, 0.1 mM hypoxanthine and 0.016 mM thymidine (Brun & Jenni, 1987). Inosine containing rEM (rEMI) was prepared by adding 0.2% inosine (Sigma Chemical Co.) to rEM. EDTA containing rEMI (rEMI-EDTA) was prepared by adding 0.2% EDTA to rEMI.

Incubators. Several incubators were used and are described as follows:

1) Candle jar (Jensen & Trager, 1977). Briefly, a lighted paraffin candle was placed in a desiccator equipped with a stopcock on the lid. The lid was sealed with silicone grease with the stopcock open to vent the heated air from within the desiccator. When the candle extinguished, the stopcock was closed and the candle jar was placed in a 37 °C incubator.

2) Gas chamber. Carbon dioxide gas was introduced to the gas chamber from one of two stopcocks of the chamber with the other stopcock open. After 5 minutes of introducing the gas, both stopcocks were closed and the gas chamber was placed in a 37 °C incubator.

3) 5% and 10% CO_2, humidified incubators (Queue CO_2 incubator, Perkinsburg, WV) and an anaerobic incubator (Forma Scientific, Marietta, OH) were used at 37 °C.

Procedures. In the whole blood (WB) culture procedure, approximately 10% parasitemic blood was prepared by mixing infected (>75% parasitemic) and noninfected blood. The mixed WB (1.2 ml) was placed in 35 mm Petri dishes, supplemented with 2% glucose and 0.2% $NaHCO_3$ (150 µl of 20% and 2% solutions, respectively), and placed in the incubator.

In the red blood cell (RBC) culture procedure, infected (>75% parasitemic) and noninfected blood were centrifuged and the plasma and buffy coat were removed. The infected and noninfected RBC's (IRBC's and URBC's, respectively) were washed twice with the same medium used in each experiment. Approximately 10% parasitized RBC's were prepared by mixing the washed IRBC's and URBC's. The 10% parasitized RBC's were suspended in the appropriate medium to yield a packed cell volume of 5%. The media used for incubation were supplemented with either 10% or 20% heat denatured (56 °C, 30 minutes) pig serum (PS) or fetal calf serum (FCS), as described in each experiment. The mixture (1.5 ml) of the 10% parasitized RBC's and the medium was placed in 35 mm diameter Petri dishes (IRBC culture), and incubated in the appropriate incubator. In EXP II-A, 60 mm diameter Petri dishes were used with 5 ml of the RBC's and medium mixture. In study II, URBC's alone were incubated (URBC culture) under the same conditions to provide a negative control.

Measurements. In study I, sequential samples were taken at various time intervals. In study II, cultures were sampled every 12 hours. Samples were taken at several points of the settled layer of the erythrocytes: RBC thin smears were prepared and stained by Wright's stain to evaluate the percentage of parasitized RBC's by counting the number of parasitized RBC's in over 400 RBC's examined.

In study II experiments, glucose concentrations of the culture media were measured (Glucose Procedure No. 510; Sigma Chemical Co.). Cumulative glucose consumption (mg/ml) was calculated by the equation:

(initial glucose concentration) - (glucose concentration of the interval sample).

In EXP II-A, lactate and pyruvate concentrations of the culture media were measured (Lactate Procedure No. 826-UV, Pyruvate Procedure No. 726-UV; Sigma Chemical Co.).

After taking the interval samples, the settled layer of RBC's and the supernatant medium were mixed by gentle swirling before returning to the incubator.

Experimental design and statistical analysis. Study I, consisting of three experiments (EXP's), was a preliminary screening of culture conditions and evaluated the effects of anticoagulants (EXP I-A), gaseous environments (EXP I-B) and media (EXP I-C) on *E. suis* growth *in vitro*.

Study II was designed to examine the *in vitro* metabolic activity of *E. suis* and further refine the *in vitro* culture

conditions. Five EXP's were designed with 3 (EXP II-A, B, C) or 5 (EXP II-D and E) replicates to allow for statistical analysis. EXP II-A examined glucose consumption, and lactate and pyruvate production in IRBC culture. EXP II-B evaluated the effect of inosine addition to the medium and EXP II-C studied the effect of EDTA addition to the medium. EXP II-D assessed serum supplementation (PS vs. FCS) of the medium and the type of incubator (5% CO_2 incubator vs. candle jar). In EXP II-E, the effect of medium refreshment (every 12 or 24 hours during 72 hours incubation) was assessed. A 10% CO_2 incubator was used in EXP II-A and 5% CO_2 incubator was used in EXP's II-B, C, D and E. Statistical analysis was done using the computer program, StatView (version 4.0, Abacus Concepts, Berkeley, CA). Student's t test was used for statistical comparison of 2 groups at each subsampling time. One way analysis of variance and multiple comparisons with Fisher's least significant difference were used for comparing 3 or more groups at each subsampling time.

Results

Study I. Preliminary Screening of Culture Conditions

Combinations of culture condition factors were applied to WB and RBC cultures in several separate experiments.

EXP I-A. Anticoagulant. The effects of different anticoagulants on the percent parasitized RBC's in WB culture using blood collected with sodium heparin, EDTA, sodium citrate or glass beads are presented in Table 2-1. Parasitized erythrocytes were observed for the longest time in heparinized blood incubated in the 10% CO_2 incubator. Few differences of the effects of anticoagulants were observed in the first 12 hours of culture. However, during the period of 12 to 24 hours of culture, a rapid decrease of the percent parasitized RBC's was observed in the EDTA anticoagulated WB culture. Heparinized and citrate anticoagulated WB cultures did not exhibit a remarkable decrease in the percentage of parasitized RBC's until after 24 hours of culture. Obvious differences were not detected between heparin and glass beads anticoagulated WB cultures. In further experiment, blood was collected with sodium heparin containing solution.

EXP I-B. Gaseous environment. The effects of different incubators and gaseous environments on the percentage of parasitized RBC's in WB and IRBC cultures are summerized in

Table 2-1. The change in the percentage of parasitized erythrocytes in whole blood culture with different anticoagulants and incubators.

		Incubation length (hours)							
Anticoagulant	Incubator	0	3	6	9	12	24	36	48
Heparin	10% CO_2	10.9	7.8	10.5	14.2	11.5	6.7	1.3	0.9
EDTA	10% CO_2	13.0	8.4	7.4	9.3	10.3	1.0	0	0
Citrate	10% CO_2	11.4	14.1	8.4	12.4	11.0	10.8	0.3	0

		Incubation length (hours)				
		0	3	8	24	48
Heparin	Candle jar	6.0	7.5	8.3	4.1	0
Beads	Candle jar	4.7	2.7	3.5	0.4	0

Table 2-2. The change in the percentage of parasitized erythrocytes in whole blood (WB) and infected red blood cell (IRBC)* cultures.

		Incubation time (hours)					
Culture	Incubator	0	2	4	6	8	24
WB	5% CO_2	11.0	11.8	10.2	15.0	17.5	5.4
WB	10% CO_2	11.0	9.2	13.3	10.9	13.6	7.5
WB	anaerobic	11.0	5.8	16.3	10.5	16.9	2.2
IRBC	5% CO_2	11.0	4.3	3.5	1.7	1.0	0
IRBC	10% CO_2	11.0	6.8	1.7	1.2	0.7	0
IRBC	anaerobic	11.0	4.9	1.2	4.4	0	0
IRBC	Candle jar	15.8	N/A†	1.3	N/A	0	0
IRBC	Gas chamber	15.8	N/A	12.2	N/A	0	0

* = IRBC culture was performed with RPMI 1640 contained 20% pig serum.
† = Not applied

Table 2-2. *E. suis* attachment to erythrocytes was observed at varying intervals during the 24 hour incubation time in WB culture with 5% and 10% CO_2, and anaerobic incubators. In IRBC culture, longer attachment was observed in either 5% or 10% CO_2 incubators than in the candle jar or the anaerobic incubator although a hihger percentage of parasitized erythrocytes were observed at 6 hours of culture with the anaerobic incubator compared to the 5 or 10% CO_2 incubators. In further experiments, 5% or 10% CO_2 incubators were used.

EXP I-C. Medium. The effects of various media on the percentage of parasitized RBC's in IRBC culture are shown in Table 2-3. For comparison, the percentage of parasitized RBC's in WB cultures performed at the same time are also shown. Parasitized RBC's were observed for at least 48 hours in IRBC culture with EM and rEM, and WB culture with glucose and $NaHCO_3$, but not in other media (RPMI 1640 or MM) or in WB culture without glucose or $NaHCO_3$ addition. The percentage of parasitized RBC's in IRBC culture with EM and rEM, and WB culture with added glucose and $NaHCO_3$ were maintained or increased at 8 hours of incubation but decreased by 24 hours. Since rEM yielded a higher percentage of parasitized RBC's at 8 and 48 hours incubation compared to EM, rEM was selected as the base medium for further experiments, in addition to heparin as the optimal anticoagulant, and 5 and 10% CO_2 incubators as the optimal gaseous environment.

Table 2-3. The change in the percentage of parasitized erythrocytes in infected red blood cell culture with various media* and whole blood culture.

	Incubation length (hours)						
Medium	0	2	4	6	8	24	48
RPMI 1640	11.0	6.8	1.7	1.2	0.7	0	N/A†
MM‡	11.0	6.1	7.9	0.5	0	0	N/A
WB-glc, $NaHCO_3$§	11.0	9.2	13.3	10.9	13.6	7.5	N/A
WB-non‖	11.0	2.5	3.9	0	0	0	N/A
EM¶	11.0	N/A	N/A	N/A	9.1	4.7	2.1
rEM#	11.0	N/A	N/A	N/A	15.8	1.4	6.8
WB-glc, $NaHCO_3$	11.0	N/A	N/A	N/A	20.4	3.1	3.9

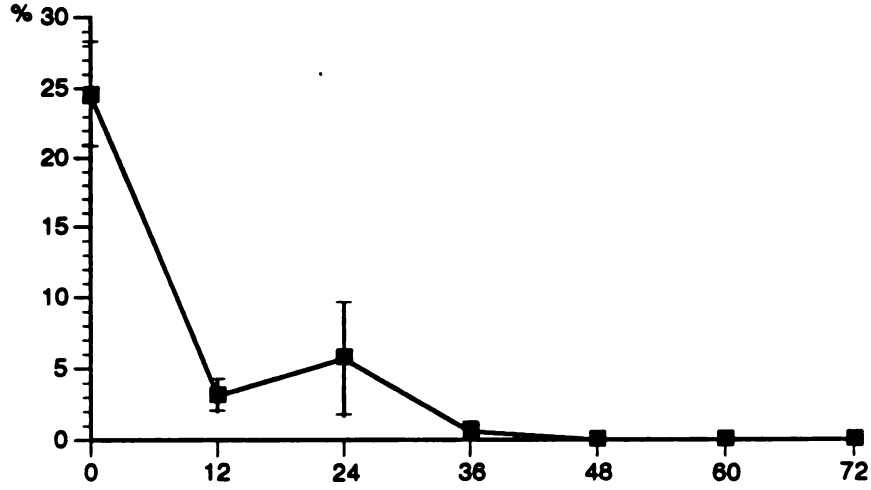
* = All media contained 20% pig serum.
† = Not applied.
‡ = Mycoplasma medium
§ = Whole blood culture with 2% glucose and 0.2% $NaHCO_3$
‖ = Whole blood culture without glucose and $NaHCO_3$
¶ = Eagle's minimum essential medium
# = Reduced Eagle's minimum essential medium with ß-mercaptoethanol

STUDY II. MEASURMENT OF *E. SUIS* METABOLIC ACTIVITY AND FURTHER SCREENING OF CULTURE CONDITIONS

EXP II-A. Glucose consumption and lactate and pyruvate production. Twenty one replicate culture dishes were prepared for each IRBC and URBC culture, with rEM containing 10% PS. In each group, three of the replicates were tested at the beginning of incubation and every 12 hours for 72 hours, and measured for the percentage of parasitized RBC's and glucose, lactate and pyruvate concentrations. Figure 2-1 illustrates the percentage of parasitized RBC's (A), and cumulative glucose consumption and lactate production (B) during incubation. During the first 24 hours of incubation, a rapid decrease in the percentage of parasitized RBC's, and a rapid increase in cumulative glucose consumption and lactate production were observed. Positive correlations were observed between the percentage of parasitized RBC's and the rate of glucose consumption and lactate production. Obvious changes in glucose consumption and lactate production in URBC culture were not detected. Pyruvate levels in IRBC and URBC cultures stayed under the detection level of the assay system throughout the experimental period.

EXP II-B. Addition of inosine to medium. The effect of providing an energy substrate for erythrocytes by adding inosine in addition to glucose was evaluated by measuring the percentage of parasitized RBC's and cumulative glucose

A. Percentage of parasitized RBC's



B. Cumulative glucose consumption and lactate production

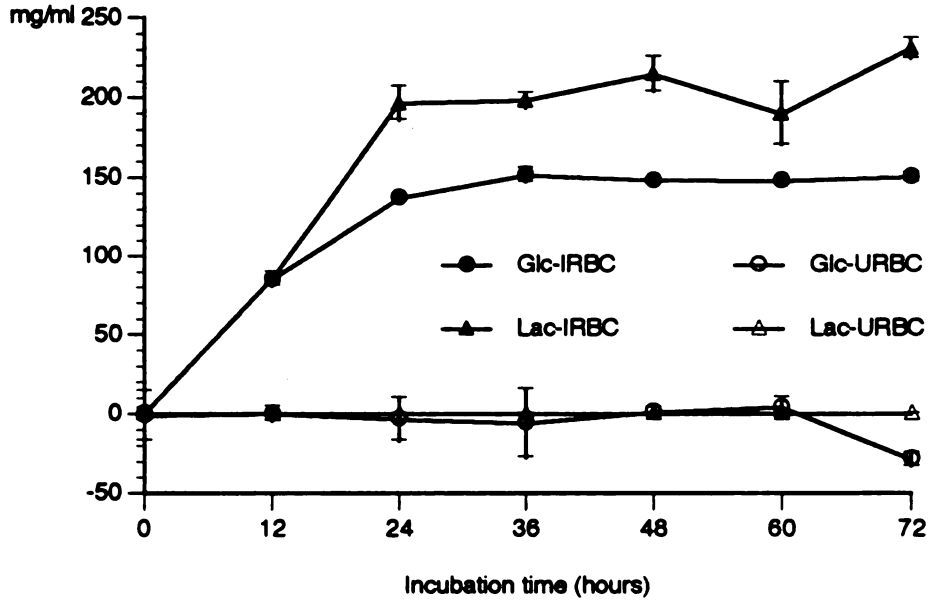


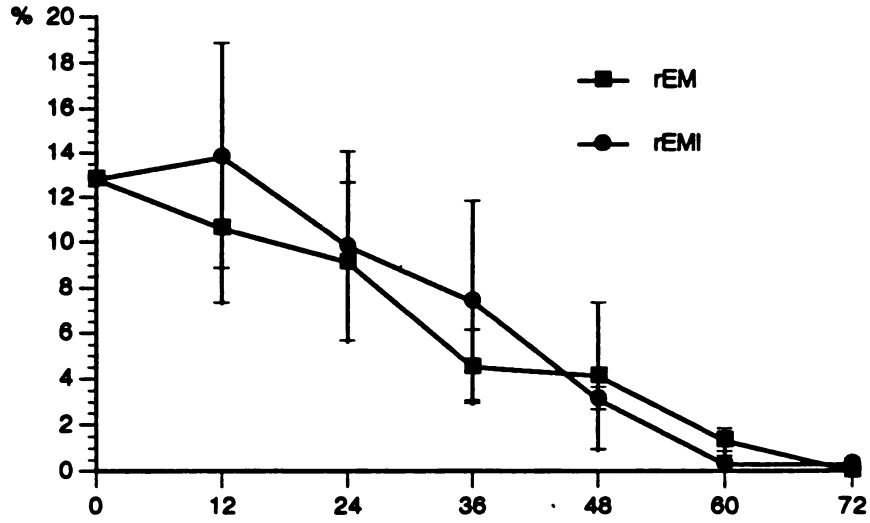
Figure 2-1. The percentage of parasitized erythrocytes (A) and cumulative glucose (Glc) consumption and lactate (Lac) production (B) in red blood cell (RBC) culture with reduced Eagle's medium. IRBC = Infected RBC culture, URBC = Uninfected RBC culture. Error bars represent standard deviations.

consumption in IRBC and URBC cultures with rEM and rEMI, both containing 10% PS (Figure 2-2). The percentage of parasitized RBC's in both media were gradually decreased during the incubation period. Cumulative glucose consumption in both media rapidly increased during the first 36 hours and remained at the same level for the rest of the incubation time. Inosine addition did not significantly change any of the parameters except for increasing the cumulative glucose consumption value at 60 hours. The major advantage of inosine addition was that erythrocyte (both infected and uninfected) integrity was better maintained in rEMI compared to rEM as detemined by the morphology in Wight's stained thin smears. Evaluating the percentage of parasitized RBC's by Wright's stained thin smears was easier with rEMI. Therefore, rEMI was used as the base media for further experiments.

Notably, the coefficients of variation (standard deviation/mean) of the percent parasitized RBC's were much greater than those of the cumulative glucose consumption. The small coeficients of variation in the cumulative glucose consumption were consistently observed in EXP's II-B, C, D and E.

EXP II-C. Addition of EDTA to medium. Under the assumption that *E. suis* loses its metabolic activity by EDTA treatment, IRBC and URBC cultures were performed with rEMI and rEMI-EDTA (both containing 10% PS) to evaluate if the apparent glucose

A. Percentage of parasitized RBC's



B. Cumulative glucose consumption

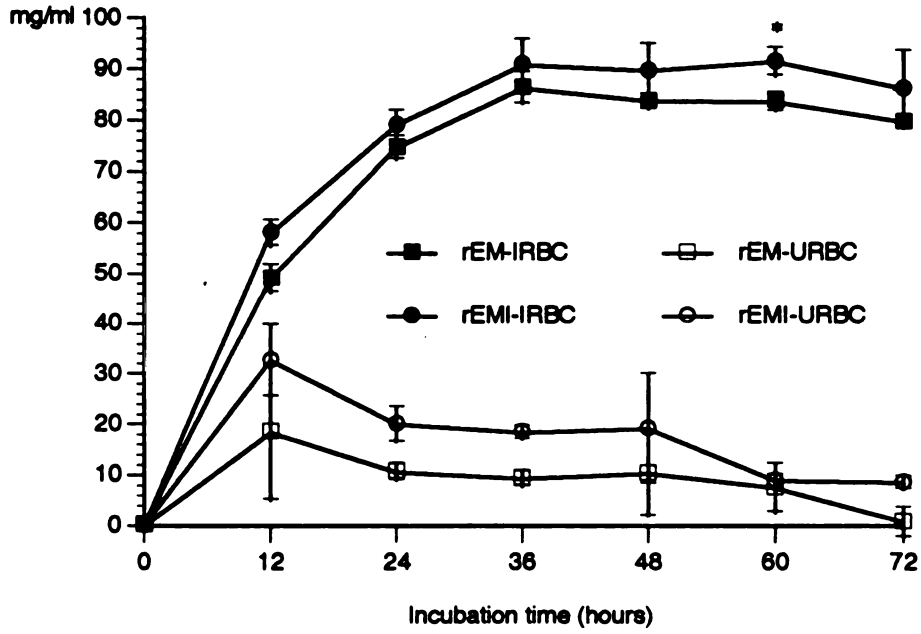
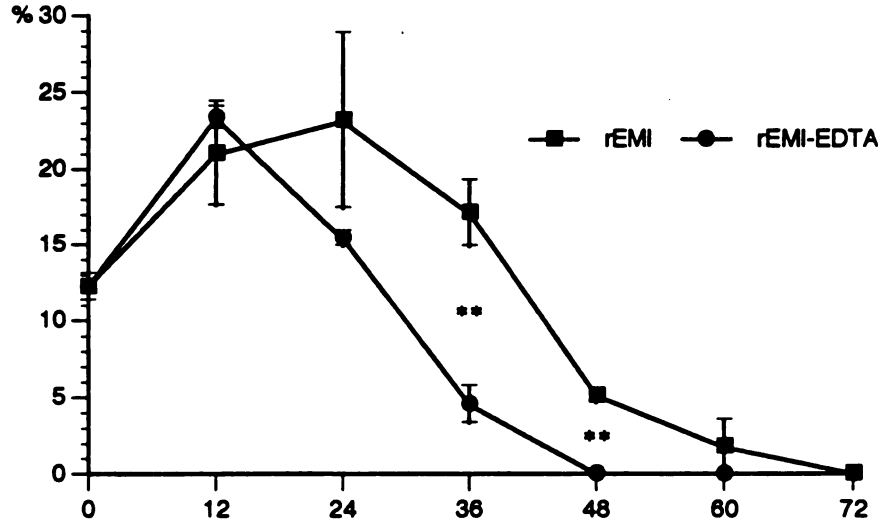


Figure 2-2. The percentage of parasitized erythrocytes (A) and cumulative glucose consumption (B) in red blood cell (RBC) culture with reduced Eagle's medium containing or not containing inosine (rEM and rEMI, respectively). IRBC = Infected RBC culture, URBC = Uninfected RBC culture. * = Significant difference ($0.01 \leq P < 0.05$) between rEM and rEMI observed in IRBC culture. Error bars represent standard deviations.

consumption is altered by the death of *E. suis*, thus determining that the source of the observed glucose consumption is the metabolic activity of *E. suis* itself rather than infected erythrocytes. Changes in the percentage of parasitized RBC's and cumulative glucose consumption, are provided in Figure 2-3. The percent parasitized RBC's in both media were initially increased at 12 hours. However, EDTA treatment caused a rapid decrease in the percentage of parasitized RBC's during 12 to 36 hours of the incubation time. The cumulative glucose consumption in IRBC culture with rEMI-EDTA was significantly lower than observed with rEMI at every sampling time except hour 0. Glucose consumption in IRBC culture with rEMI-EDTA ceased at 24 hours, since the cumulative glucose consumption stayed at the same level from 24 to 72 hours of incubation time. Considering that the number of *E. suis* attached per infected erythrocyte did not increase during the experimental period, a positive correlation was noted between the number of *E. suis* attached to RBC's and the rate of glucose consumption.

The cumulative glucose consumption values of the rEMI-EDTA in URBC culture gradually decreased over the experimental period. Those values were significantly less than those of the rEMI in URBC culture during 12 to 72 hours of incubation.

A. Percentage of parasitized RBC's



B. Cumulative glucose consumption

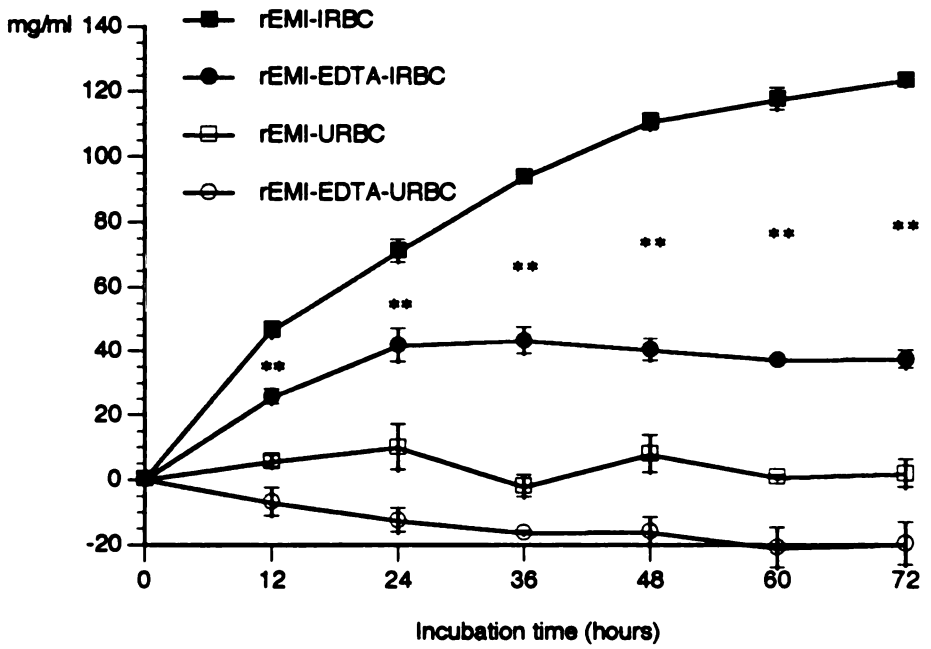


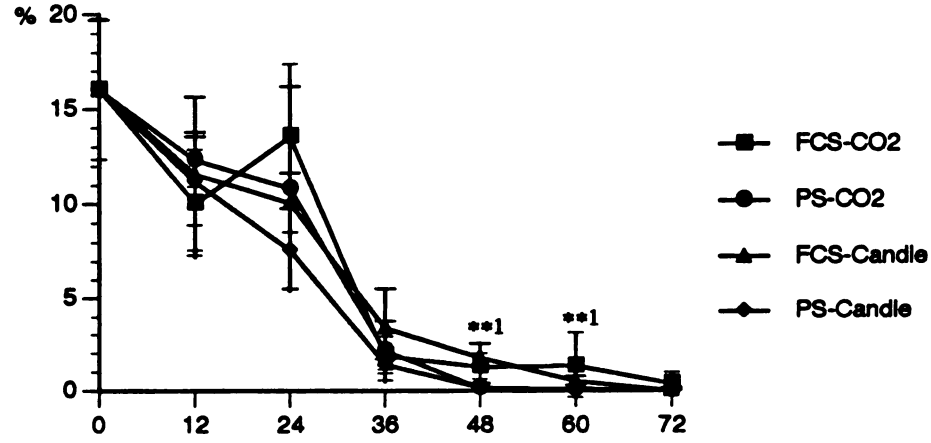
Figure 2-3. The percentage of parasitized erythrocytes (A) and cumulative glucose consumption (B) in red blood cell (RBC) culture with reduced Eagle's medium containing inosine and EDTA (rEMI-EDTA) and reduced Eagle's medium containing only inosine (rEMI). IRBC = Infected RBC culture, URBC = Uninfected RBC culture. ** = Significant difference ($P<0.01$) between rEMI and rEMI-EDTA in IRBC culture. Error bars represent standard deviations.

EXP II-D. Selection of serum and incubator. The effect of PS versus FCS addition to the medium, and incubating in 5% CO_2 incubator versus a candle jar were evaluated. Changes in the percentage of parasitized RBC's in each combination of serum and incubator is shown in Figure 2-4 A. Any indications of the organisms' multiplication or significant differences between the combinations of sera and incubators were not observed, except at the time when the percentage of parasitized RBC's was very low. Significant differences of glycolytic activity were detected with the different sera (Figure 2-4 B). A significantly greater cumulative glucose consumption was observed in rEMI containing FCS compared to PS from 24 to 72 hours. Few differences were observed between the CO_2 incubator and the candle jar (Figure 2-4 C). The cumulative glucose consumption values in URBC cultures gradually decreased over the experimental period.

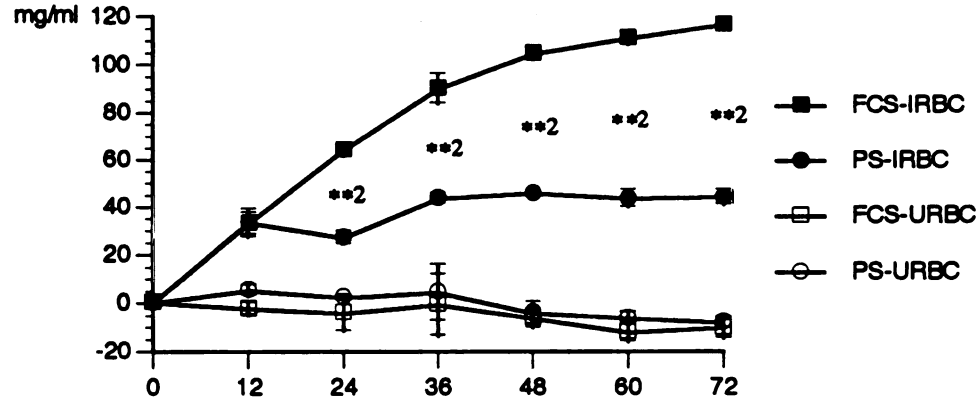
EXP II-E. Refreshment of medium. Using the optimal medium (rEMI containing 10% FCS) and incubator (5% CO_2) based on the results of the previous experiments, IRBC cultures composed of 3 groups of 5 replicates were prepared. As a negative control, URBC cultures were also prepared with 2 replicates in each group. In groups 1 and 2, old medium in the Petri dishes was removed and fresh medium was added every 12 and 24 hours, respectively. In group 3, medium was not changed over the experimental period. Changes in the percentage of parasitized RBC's and cumulative glucose consumption are

Figure 2-4. The percentage of parasitized erythrocytes (A) and cumulative glucose consumption (B and C) in red blood cell (RBC) culture with reduced Eagle's medium with inosine (rEMI) containing 10% fetal calf serum (FCS) or pig serum (PS), incubated in a 10% CO_2 incubator or a candle jar. B. Cumulative glucose consumption in IRBC and URBC culture with rEMI containing 10% FCS or PS, incubated in a 10% CO_2 incubator. C. Cumulative glucose consumption in IRBC and URBC culture with rEMI containing 10% FCS, incubated in a 10% CO_2 incubator or a candle jar. **'s = Significant differences ($P<0.01$) between FCS-CO_2 and PS-CO_2 (**1), between FCS-IRBC and PS-IRBC (**2), and between CO_2-IRBC and Candle-IRBC (**3). Error bars represent standard deviations.

A. Percentage of parasitized RBC's



B. Cumulative glucose consumption - Serum



C. Cumulative glucose consumption - Incubators

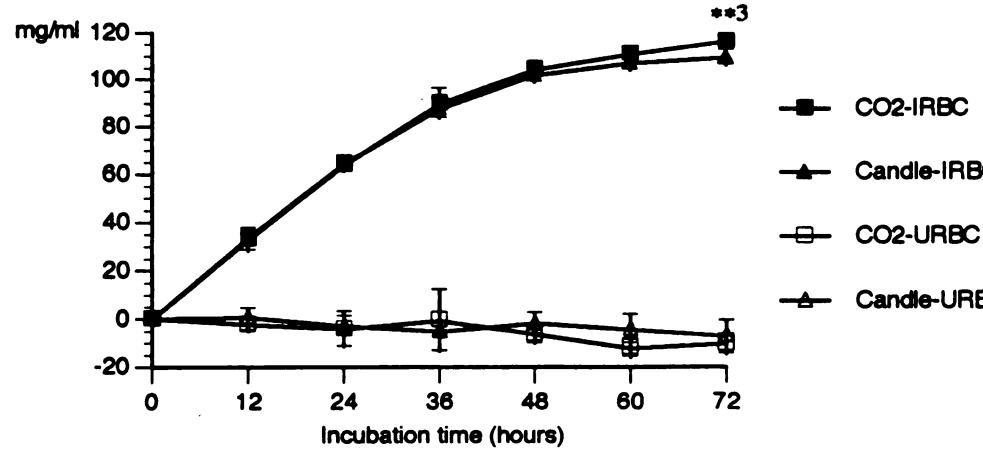
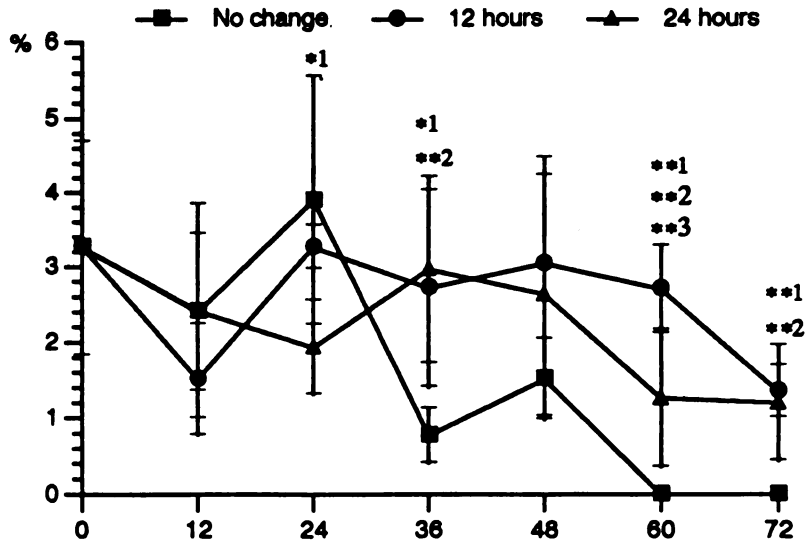


Figure 2-4.

shown in Figure 2-5. The percentage of parasitized RBC's in groups 1 and 2 remained at the same level, although the standard deviations at each subsampling time were great. In group 3, there was a gradual decrease in the percentage of parasitized RBC's, declining to 0% at 60 hours. Significant differences were observed at several sampling times between groups 1 and 3, and between groups 2 and 3. Refreshment of medium (groups 1 and 2) resulted in higher cumulative glucose consumption at sampling times from 36 to 72 hours. Similar effects on the percentage of parasitized RBC's and cumulative glucose consumption were observed between groups 1 and 2, although group 1 exhibited significantly greater values at 60 hours in the percentage of parasitized RBC's and at 72 hours in the cumulative glucose consumption compared to group 2.

A. Percentasge of parasitized RBC's



B. Cumulative glucose consumption

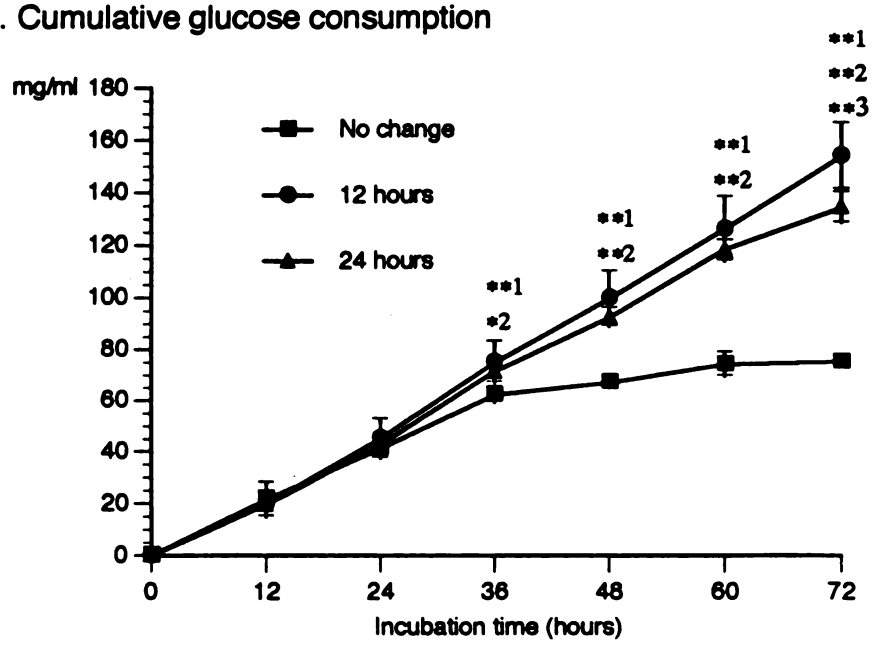


Figure 2-5. The percentage of parasitized erythrocytes (A) and cumulative glucose consumption (B) in infected red blood cell (IRBC) culture with medium refreshment every 12 hours and 24 hours, and without changing medium. * and ** = Significant differences (*=0.01≤P<0.05, **=P<0.01) between "No change" and "12 hours" (*1, **1), between "No change" and "24 hours" (*2, **2) and between "12 hours" and "24 hours" (3). Error bars represent standard deviations.**

Discussion

The successful cultivation of parasitic protozoa requires optimum biophysical growth conditions (temperature, pH, O_2 and CO_2 concentrations) and the nutritional supplementation of amino acids, vitamins, carbohydrates, and sometimes blood or blood derivatives (Enders, 1988). Successful *in vitro* culture systems have been described for *Plasmodium* and *Babesia* (Jensen, 1983; Levy & Ristic, 1983) after a long history of attempts and failures to select the optimum culture conditions. One of the most fascinating findings in the history of *Plasmodium in vitro* culture is that the parasite was an obligate microaerophile, and favored lower oxygen tensions (5 - 15%) and higher carbon dioxide tensions (3 - 5%) than provided by air (Jensen, 1983; Scheibel et al., 1979; Trager, 1941). With this evidence, Trager and Jensen (1976) introduced the Petri dish-candle jar method for *in vitro* cultivation of *Plasmodium falciparum*, using RPMI 1640 as a base medium. This technique was applied to the *in vitro* culture of *Anaplasma marginale*, and *in vitro* multiplication and maintenance of infectivity for up to 33 days was achieved (Kessler & Ristic, 1979; Kessler et al., 1979; Mazzola & Kuttler, 1980).

In study I, preliminary screening of culture conditions was based on modifications of the culture procedure developed for *Plasmodium falciparum* (Jensen & Trager, 1977). However in RPMI 1640 systems, parasitized RBC's were no longer

visible by 24 hours of incubation, regardless of the types of incubator. Incubating whole blood with glucose and sodium bicarbonate yielded superior results compared to RPMI 1640 systems as parasitized RBC's were observed for up to 48 hours. Since *E. suis* is attached externally to RBC's or lives free in the plasma, *E. suis* is exposed to extracellular conditions, unlike malaria parasites which reside inside RBC's. *In vitro* culture systems for other extracellular blood parasites such as salivarian trypanosomes, had been developed using Eagle's minimum essential medium. For example, *Trypanosoma brucei* was originally cultivated in feeder layer systems using RPMI 1640 or Eagle's medium. Later, cell free semi-defined medium based on Eagle's medium was established by providing reducing conditions using β-mercaptoethanol instead of feeder layer cells (Brun & Jenni, 1987). In the experiment of medium selection (EXP I-C), reduced and non-reduced EM were applied to the Petri dish system, and both conditioned medium yielded acceptable results for *in vitro* maintenance of *E. suis* attachment on RBC's during cultivation.

Glucose consumption, and lactate and pyruvate production by *E. suis* was expected based on the observation of hypoglycemia in acutely infected animals, and decreased glucose and increased lactate concentration during *in vitro* incubation of EDTA anticoagulated blood with supplemental glucose (Heinritzi et al., 1990a; Heinritzi et al., 1990b). In the rEM culture system, a rapid decrease in glucose levels

and a rapid increase in lactate levels in the culture medium were observed (EXP II-A). These results could have been caused by parasite metabolism alone and/or enhanced erythrocyte metabolism caused by *E. suis*. It is known that glucose consumption for energy production by pig erythrocytes is extremely low, because pig erythrocytes lack a functional glucose transporter (Harvey, 1989; Rivkin & Simon, 1965). It has been observed that the amount of ATP possessed by pig erythrocytes decreases by 50% at 8 hours of *in vitro* incubation, regardless of the presence or absence of glucose (Kim & McManus, 1971). Although pig erythrocytes have the capacity for complete glycolysis, glucose uptake is normally limited by the low permeability to glucose (Kim & McManus, 1971), suggesting that pig erythrocytes could consume glucose if glucose enters into pig erythrocytes by some other means. Electronmicrographs have suggested that *E. suis* parasitism causes membrane deformation of erythrocytes (Zachary & Basgall, 1985). Therefore, it could be hypothesized that *E. suis* infection may enhance erythrocyte permeability for glucose by disturbing erythrocyte membrane structure, thus the infected erythrocytes may consume more glucose than noninfected erythrocytes. Parasite induced, increased glucose permeability has been observed in *Babesia bovis* infected erythrocytes (Upston & Gero, 1990). EXP II-C was conducted to determine if *E. suis* actually consumes glucose during *in vitro* culture by evaluating if *E. suis* death affects the apparent glucose consumption *in vitro*. In this

experiment, the assumption was made that *E. suis* is killed by EDTA treatment, thus losing its glycolytic activity. The effect of EDTA was observed in EXP I-A, in which *E. suis* could not maintain its attachment to RBC's in the EDTA anticoagulated blood when compared to heparin or citrate anticoagulated blood. Also, a previous study found that *E. suis* lost its infectivity with EDTA treatment (Hall et al., 1988). In EXP II-C, EDTA treatment showed profound negative effects on *E. suis* maintenance of RBC attachment and glycolytic activity, and a positive correlation between the number of attached *E. suis* and the rate of glucose consumption was observed. This indicates that glucose consumption in the culture system was primarily due to *E. suis* glycolysis.

The possibility that the glycolytic activity of erythrocytes was enhanced by *E. suis* infection was unsubstantiated by observation that the integrity of infected erythrocytes incubated with rEM (without inosine) was not maintained sufficiently. If the glycolytic activity of infected erythrocytes was enhanced, infected erythrocytes were supposed to sufficiently acquire energy from glycolysis for maintaining their membrane integrity. Therefore, poor maintenance of membrane integrity of infected erythrocytes under the presence of sufficient glucose suggested that the glycolytic activity of infected erythrocytes was not sufficiently enhanced to maintain membrane integrity.

In EXP II-C, significant differences were also observed in the cumulative glucose consumption in the URBC culture between rEMI and rEMI-EDTA. EDTA is known to chelate heavy metal ions, such as Ca^{2+} and Mg^{2+}. In the glycolytic pathway, magnesium ions serves as a cofactor for many enzymes. It could be assumed that EDTA may inhibit glycolysis. Therefore, the observed difference in URBC culture between rEMI and rEMI-EDTA could indicate that pig erythrocytes consumed some glucose, and glycolysis of pig erythrocytes was inhibited by EDTA. On the other hand, the blocking of glycolysis by EDTA may explain in part that *E. suis* was killed by EDTA treatment.

In study I and study II-A, erythrocytes observed in the thin smears, especially in IRBC culture after 24 hours of incubation, exhibited blunt membranes. This observation suggested that the erythrocyte membranes became fragile because of *E. suis* parasitism and/or *in vitro* incubation. In feline haemobartonellosis, it has been observed that the osmotic fragility of *Haemobartonella felis* infected erythrocytes was increased as a result of decreased erythrocyte lipid concentration (Maede, 1980). The cause of decreased lipids concentration in erythrocyte membrane was not clear, however several possibilities were listed: 1) the erythrocyte membrane may be altered by lipolytic enzymes produced by *H. felis*; 2) *H. felis* may utilize the erythrocyte lipids for its own nutrition; and 3) inadequate energy supply to erythrocytes because of hypoglycemia caused by *H. felis*

infection may cause lipid loss (Cooper & Jandle, 1969; Maede, 1980). As discussed above, pig erythrocytes can not acquire glucose sufficiently for its sole energy source. To provide energy to pig erythrocytes, the medium was supplemented with inosine (EXP II-B). Pig erythrocytes may use the nucleoside inosine as an energy source by the following proposed mechanism:

inosine + Pi → ribose-1-P + hypoxanthine

ribose-1-P → ribose-5-P

The ribose-5-P is integrated into the pentose phosphate shunt in the glycolytic pathway. Remarkable effects of inosine addition to the medium on the maintenance of *E. suis* attachment on RBC's and glycolytic activity were not detected in EXP II-B; however, the *in vitro* maintenance of erythrocyte integrity was improved by addition of inosine to the medium and erythrocyte membranes in the thin smears remained sharp for at least 72 hours of incubation. Therefore, inosine addition facilitated easier and possibly more accurate evaluation of the percentage of parasitized RBC's in thin smears.

In EXP II-D, the effect of serum and gaseous environment were evaluated using the rEMI system. In the various *in vitro* culture systems, serum contributes various undefined nutrients including growth factors, vitamins and ions (Brun & Jenni, 1987). In the present experiments, FCS produced a profound positive effect on *E. suis* glycolytic activity. FCS is known to contain various growth factors which are required

for fetal calf growth. It appears that these factors also have beneficial effects on *E. suis* growth *in vitro*. The candle jar used for *Plasmodium falciparum* was re-evaluated in the same experiment using the rEMI system; however, no improvements were observed.

In the final experiment (EXP II-E), medium refreshment at either 12 or 24 hour intervals during incubation prolonged attachment of *E. suis* on erythrocytes and improved glucose consumption. Refreshment of medium provides additional nutrients and removes toxic metabolites. Production of lactate, one of the major metabolites in the culture system, might cause negative effects on *E. suis* activity by altering the pH of the medium. The negative effect of decreased pH as a result of lactate production was discussed in the *in vitro* culture system for malaria parasites (Jensen, 1983).

In EXP's II-B through II-E, the coefficients of variation from cumulative glucose consumption data were much less than the percentage of parasitized RBC's data. This finding suggested measuring glucose consumption was a more precise method for evaluating *E. suis* activity in the developed culture system. The subsamples for thin smears were taken from the several points of the settled erythrocyte layer at the bottom of the culture dishes. Therefore, if *E. suis* grows as a cluster during cultivation, the percentage of parasitized RBC's was highly dependent on the location from which subsamples were taken. In addition, Henry (1979) described that artifacts of red cells in blood smears were

difficult to distinguish from *E. suis* bodies in many cases. Especially, small forms of the organism, presumably as a result of budding multiplication, were almost impossible to distinguish from stain deposit or stained dust particles. Measuring glucose consumption has another advantage for evaluating *E. suis* activity *in vitro*. Glucose could be consumed by *E. suis* free in the medium as well as *E. suis* associated with erythrocytes. Therefore, glucose consumption reflects the activity of *E. suis* both associated with RBC's and free in the medium, while the percent parasitized RBC's did not account for *E. suis* free in the medium.

The negative values observed in the cumulative glucose consumption in URBC cultures, especially at the longer incubation period, are probably a reflection of the medium evaporation. Since glucose does not evaporate from the medium, the relative glucose concentration in the medium increases as water evaporated. In EXP II-B, water evaporation in each Petri dish between subsampling times was measured by weighing Petri dishes before and after incubation. The weight of Petri dishes decreased by 0.8% of the medium weight in each 12 hour interval with negligible variation between Petri dishes, indicating glucose concentration in each Petri dish was equally concentrated by 0.8%. Since EXP II-B was assumed to be representative for the effect of water evaporation, the weight of the Petri dishes were not measured in other experiments.

Throughout the experiments, an increase in the percentage of parasitized RBC's was occasionally observed during the first 12 hours of incubation. It is not certain if this observation was a reflection of active cell to cell transmission or simply a manifestation of an infection initiated *in vivo*. As a general tendency, the number of parasitized RBC's gradually decreased during the incubation period, except EXP II-E in which a low percentage of parasitized RBC's were maintained by refreshment of the medium, throughout the incubation period.

Ichijo et al. (1982) observed a 35% parasitemia in bone marrow of *E. ovis* infected sheep while the parasitemia in peripheral blood was 4%, and suggested that bone marrow may be a main site for parasite multiplication. According to this finding, bone marrow culture may be a good alternative for the proposed RBC-Petri dish system. Attempts at *in vitro* culture with bone marrow were reported with *Anaplasma marginale* with bovine bone marrow (Hruska et al., 1968), and rabbit bone marrow (Marble & Hanks, 1974a; Marble & Hanks, 1974b). However, clear evidence of active replication of *A. marginale* in the bone marrow systems was not obtained. In an electron microscopic study of bone marrow obtained from an *E. suis* parasitemic pig, we were unable to detect *E. suis* parasitism on immature red cells (unpublished data). Therefore, the value of bone marrow culture for *E. suis* is questionable.

In the RBC culture procedure, erythrocytes settled at the bottom of the Petri dishes during incubation period, probably in the first couple of hours. It is suspected that *E. suis* are immobile organisms because no structures associated with mobility, such as pili, flagella or actin, were observed in electronmicroscopic studies (Pospischil & Hoffmann, 1982; Zachary & Basgall, 1985). Therefore, an opportunity for *E. suis* to encounter new erythrocytes was limited in the developed culture system. It may be advantageous to apply gentle agitation during cultivation. Since *E. suis* is an extracellular organism living in blood, gentle agitation may mimic its *in vivo* environment.

The proposed RBC culture is the first description of an *in vitro* culture system for *E. suis* using semi-defined medium. Although continuous cultivation was not achieved, the system was successful for short term maintenance of *E. suis* attachment on erythrocytes and measurement of *E. suis* glycolytic activity. The 72 hour time period appears to be sufficient for evaluating anti-*E. suis* compounds *in vitro*, as demonstrated in EXP II-C using EDTA. Furthermore, the system could be used for protein and DNA analysis by radiolabeling the organism using radiolabeled nutrient, since metabolic activity can be maintained in the system (refer to Chapter III). Further investigation of the *in vitro* requirements of *E. suis* growth could lead to the continuous *in vitro* propagation of *E. suis* and establishment of *in vitro* experimental systems for various purposes.

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CHAPTER 3

LACK OF SPECIFICITY OF THE INDIRECT HEMAGGLUTINATION ASSAY FOR SWINE EPERYTHROZOONOSIS AND IDENTIFICATION OF *EPERYTHROZOON SUIS* SPECIFIC ANTIGENS

Abstract

The indirect hemagglutination assay (IHA) for swine eperythrozoonosis was evaluated for specificity in detecting *E. suis* specific antibodies by analyzing the effects of incubation temperature and absorption of immune sera with normal pig erythrocytes, and by using IHA antigens prepared from infected and noninfected blood in the IHA, ELISA and western blots. With incubation at 37 °C instead of 4 °C used in the standard IHA procedure, 77% of tested sera showed either an increase or decrease in titer compared to their respective standard IHA titers. More than two fold titer decreases were observed in standard IHA positive (>1:80) and suspicious (1:40) sera, indicating an active role of cold temperature reacting antibodies in IHA. With preincubation of test sera with normal pig erythrocytes at either 4 or 37 °C, the titers of standard IHA positive sera tended to decrease, regardless of preincubation temperature, indicating an active role of autoantibodies in IHA. No differences were observed in either the IHA titers or ELISA optical densities between Es^+ (*E. suis* infected) and Es^- (*E. suis* noninfected) antigen preparations. *E. suis* specific antigens were not readily detected in the IHA antigen preparations by western blotting, indicating antibodies measured in IHA were directed mainly at host proteins.

Three methods were investigated for identification and partial purification of *E. suis* antigens: 1) high speed

centrifugation of *E. suis* dissociated from erythrocytes by EDTA; 2) high speed centrifugation and Percoll separation of *E. suis* free in plasma; and 3) differential centrifugation of *E. suis* dissociated from hemolysates. Isolated *E. suis* were solubilized and analyzed by SDS-PAGE and Western blotting. With the first 2 methods, *E. suis* specific antigens were not readily observed. With the third method, frozen erythrocytes were thawed and centrifuged at 100,000g. The pellet was resuspended in PBS and sequentially centrifuged at 500g, 10,000g and 100,000g. After each centrifugation, the pellet was removed and analyzed. A number of *E. suis* specific antigens were readily observed, especially in the 100,000g sediment. Antigen bands at 24, 47, 71, 79, 81, 90, 106 kd were consistently observed in western blots reacted with sera obtained from various pigs and at different post-infection times, and by autoradiography using an antigen prepared from ^{35}S labeled *E. suis*. Some antigen bands were only recognized by sera collected during the late stage of infection. Sizes of the late appearing antigens and time recognized varied among the pigs. Host protein inclusion was least in the 100,000g sediment.

Introduction

Swine eperythrozoonosis is caused by the rickettsial organism, *Eperythrozoon suis*. The disease is characterized by acute ictero-anemia and is associated with impaired weight gains and reproductive performance (Henry, 1979). Swine eperythrozoonosis has been reported world wide including North and South America, Africa, Europe and Asia (Henry, 1979; Hsu et al., 1990; Sisk et al., 1980; Smith, 1977; Smith, 1981).

Prior to 1970, the diagnosis of swine eperythrozoonosis was based on herd and individual animal histories describing ictero-anemia, demonstration of *Eperythrozoon* bodies in blood smears, and the complement fixation test (Splitter, 1958). *E. suis* was considered primarily a pathogen of feeder pigs, causing acute icteroanemia, with low morbidity and high case mortality. Detection of the organism by blood smears provides a definite diagnosis; however, this method is complicated by difficulties in distinguishing *E. suis* bodies from artifacts in blood smears (Henry, 1979). The majority of *E. suis* infections are believed to be subclinical and parasitemia is only detected in acutely affected animals.

Smith and Rahn (1975) developed an indirect hemagglutination assay (IHA) for measuring *E. suis* antibodies. IHA positive, non-parasitemic animals have been observed routinely and are considered to be carriers of *E. suis*. IHA has become the standard diagnostic test for

detecting *E. suis* antibodies. The IHA antigen is prepared by glutaraldehyde attachment of a partially purified antigen preparation made from the plasma of parasitemic pigs to sheep erythrocytes. Serial dilutions of sera are reacted with the treated sheep erythrocytes to determine the titer. A positive IHA titer is associated with IgM antibodies, correlated with anemia but not with development of detectable parasitemia, and indicates either a recent or persistent/chronic infection (Zachary & Smith, 1985).

Hoffmann et al. (1981) classified the anemia in swine eperythrozoonosis as an autoimmune hemolytic anemia and autoantibodies against erythrocytes were thought to play an important role in anemia development. Anti-erythrocytic antibodies have been suggested by several observations such as spontaneous agglutination of infected blood especially at cold temperatures, increased sedimentation rate of infected blood (Doyle, 1932; Robb, 1943; Splitter & Williamson, 1950) and positive Coomb's tests (Hoffmann et al., 1981). Recently, Schmidt et al. (1992) have isolated a cold agglutinin from erythrocytes of infected animals. Biochemical analysis using double immunodiffusion, polyacrylamide gel electrophoresis and western blotting with purified IgM and anti-pig IgM revealed that the isolated cold agglutinins were IgM antibodies.

The associations of IgM with IHA reactive antibody, IHA titer with anemia development and anemia development with IgM cold agglutinin production, plus the likelihood that the IHA

antigen preparation contains host derived antigens, suggest the possibility that IgM antibodies detected in the IHA test may be directed at host erythrocyte antigens as well as *E. suis* antigens, thus potentially reducing test specificity. Furthermore, Smith (1981) reported that IHA negative pigs could be carriers because parasitemia was observed in IHA negative pigs after splenectomy, indicating that the test may also lack sensitivity for detecting chronically infected carriers.

Recently, enzyme-linked immunosorbent assays (ELISA's) have been developed to detect *E. suis* specific antibodies and improve test sensitivity for detecting low grade infections (Hsu et al., 1992; Schuller et al., 1990). The coating antigens used in these assays were crude preparations from infected erythrocyte lysates and contained host erythrocyte antigens. Again, considering the autoantibody production induced by *E. suis* infection, the presence of host proteins in ELISA coating antigens may interfere with test results.

The following studies were initiated to evaluate the specificity of the IHA for detecting *E. suis* specific antibodies and to develop and evaluate improved antigen preparations to facilitate improvements in serodiagnostic tests for swine eperythrozoonosis.

Materials and Methods

Organism. *E. suis* was initially isolated from an inappetant, febrile boar housed at the Michigan State University Swine Research Center. A diagnostic CBC revealed that the boar was parasitemic and anemic. This *E. suis* isolate has been maintained by passage in splenectomized pigs. Infected blood has been cryopreserved by adding 6.7% and 10% (v/v) of 40% polyvinyl pyrrolidone (PVP) and glycerol, respectively, and freezing in liquid nitrogen.

Animals and experimental infection. Cross bred pigs were 6- to 13-weeks-old at inoculation. All animals were splenectomized using standard surgical techniques 1 to 2 weeks prior to inoculation and determined to be *E. suis* noninfected by no evidence of any temperature elevation or development of parasitemia before inoculation. Ten milliliter of frozen infected blood was thawed in a 37 °C water bath and inoculated intravenously and/or intramuscularly into splenectomized pigs. Clinical signs and rectal temperatures were assessed daily for monitoring disease development. When the rectal temperature exceeded 40 °C, blood was collected in a vacutainer tube (Beckton Dickinson Vacutainer Systems, Rutherford, NJ) containing ethylenediamine tetraacetic acid (EDTA) and examined for parasitemia by Wright's stained thin smear. When the febrile phase lasted for more than 3 days or if an animal exhibited

severe depression, intramuscular treatment with oxytetracycline (20 mg/kg body weight; Liquamycin LA 200, Pfizer, New York, NY) was initiated to avoid mortality.

Serum samples. Serum was harvested from blood obtained from experimentally infected pigs before inoculation and weekly thereafter. Serum samples (field sera) submitted to the Michigan Department of Agriculture for regulatory purposes were kindly provided by Dr. Richard Gatzmeyer. In addition, serum samples submitted to the Michigan Animal Health Diagnostic Laboratory for swine eperythrozoonosis testing were used.

The immune reference serum was a pool of sera obtained from blood collected at 2 to 8 weeks post-infection from experimentally infected, splenectomized pigs. The nonimmune reference serum was obtained from blood of a known noninfected, intact pig.

Antigen preparation. Antigens were prepared by four different methods:

1) Method IHA antigen: IHA antigen preparation method followed the procedure of Smith and Rahn (1975). Briefly, parasitemic blood was collected using a sodium citrate solution, and plasma was separated by centrifugation. Globulins were removed from the plasma by precipitation with saturated ammonium sulfate (1:1) and the precipitate was discarded. The supernatant was dialyzed against saline

borate buffer (pH 8.0) for 12 hours and concentrated to 1/5 of the initial plasma volume by Amicon ultrafiltration using an XM 50 filter (Amicon Division, W. R. Grace & Co., Danvers, MA). Albumin was removed from the concentrated preparation by precipitation with three times its volume of 0.1 M tris buffer (pH 8.0) containing 0.4% ethodin (6,9-diamino-2-ethoxyacridine lactate). The supernatant was again dialyzed and stored at -80 °C. Commercially available IHA antigen (Dr. A. R. Smith, University of Illinois, Urbana-Champaign, IL) was used as a positive control.

2) Method EDTA-Es (Isolation of *E. suis* dissociated from erythrocytes by EDTA): Blood collected into sodium heparin solution (30 U/ml) was centrifuged at 275g for 20 minutes, and the plasma and buffy coat were removed. The sediment (erythrocytes) was mixed with phosphate buffered saline (PBS) containing 1% EDTA (EDTA-PBS), incubated for one hour at 37 °C with gentle rocking, and centrifuged at 500g for 20 minutes. The supernatant was collected and centrifuged at 20,000g for 60 minutes at 4 °C. The resulting pellet was solubilized by 3 times freeze-thawing in Tris buffer (pH 8.0) containing 5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40 and 0.1% sodium dodecyl sulfate (SDS), and stored at -80 °C.

3) Method Plasma-Es (Isolation of *E. suis* free in plasma): A) Blood collected into sodium heparin solution (30 U/ml) was incubated at 37 °C for one hour with gentle rocking and then centrifuged at 500g for 20 minutes. Plasma was

collected and centrifuged at 20,000g for 60 minutes at 4 °C. The resulting pellet was solubilized and stored at -80 °C. B) The pellet obtained by Method Plasma-Es-A, was mixed with isotonic Percoll (70% in final concentration; Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged at 60,000g for 15 minutes. Visible bands were collected and examined for the presence of *E. suis* by electron microscopy using phosphotungstic acid negative stain.

4) Method D'spin-Es (Isolation of *E. suis* by differential centrifugation of hemolysates): Blood collected into citrate-phosphate-dextrose solution containing 0.2% inosine was centrifuged at 500g for 20 minutes, and the plasma and buffy coat were removed. Erythrocytes were washed 3 times with Eagle's minimum essential medium containing 0.2% inosine (EMI). After the final wash, erythrocytes were suspended in the same volume of EMI containing 5% fetal calf serum (FCS). The suspension was mixed with 6.7% and 10% (v/v) of 40% PVP and glycerol, respectively, and stored at -80 °C. The frozen erythrocytes were thawed and centrifuged at 100,000g for 40 minutes. A small aliquot of the pellet was solubilized and stored at -80 °C (designated as thaw antigen). The remainder of the pellet was resuspended in PBS and sequentially centrifuged at 500g for 20 minutes and at 10,000g and 100,000g for 30 minutes each. After each centrifugation, the pellet was removed, solubilized and stored at -80 °C (designated as 500, 10K and 100K antigens, respectively).

Antigens were prepared either from infected (>75% parasitemia, designated as Es^+, *E. suis* positive antigen) or noninfected blood (Es^-, *E. suis* negative antigen) by each preparation Method. In addition, the 100K antigen described in Method D'spin-Es was prepared from the blood of one pig collected: 1) before splenectomy, 2) after splenectomy and before inoculation of *E. suis*, 3) at the first parasitemic phase, 4) after the first parasitemic phase during which parasitemia was not detectable, 5) at the second parasitemic phase, and 6) after the second parasitemic phase. The protein concentrations of the antigen preparations were measured by the Bio-Rad protein assay (Bio-Rad, Richmond, CA).

Preparation of E. suis sensitized sheep erythrocytes (S-RBC) and glutaraldehyde treated sheep erythrocytes (G-RBC) for IHA. Five milliliter of sheep blood was collected with an equal volume of Alsever's solution and the erythrocytes were washed 3 times with PBS (pH 7.2). A half milliliter of packed erythrocytes were suspended in 9.5 ml of PBS in a 50-ml beaker and stirred on a magnetic stirrer. While stirring, 24.7 mg of IHA antigen was added, followed by dropwise addition of 1 ml of 2.5% glutaraldehyde. The mixture was stirred for one hour, then 1 ml of 2% ovalbumin was added and the mixture was stirred for another 30 minutes. S-RBC were washed 3 times and suspended in 9 times its volume of PBS and stored in liquid nitrogen. For use in the IHA, the thawed

10% S-RBC solution was diluted 1:20 with PBS. G-RBC were prepared identical to S-RBC, except no antigen was added. G-RBC was used as a 10% solution.

Indirect hemagglutination assay (IHA). Several IHA procedures with modifications of the Smith and Rahn (1975) method were performed:

1) Standard IHA: Test sera were heat inactivated at 56 °C for 30 minutes. To 100 µl of inactivated serum, 0.85 ml of PBS and 50 µl of the 10% G-RBC solution were added and the mixture was incubated for 15 minutes at 25 °C. The mixture was then centrifuged at 500 g for 10 minutes and 50 µl of the supernatant was transferred to a round bottom 96 well microplate. The transferred sera was serially diluted (two fold) with 1% heat inactivated rabbit serum in PBS, with a starting dilution of 1:10. The test antigen, 50 µl of 0.5% S-RBC solution, was added to each well. The plate was covered and incubated at 4 °C over night.

The IHA titers on individual samples were interpreted as follows: 1:80 or more, positive; 1:20 or less, negative; and 1:40, suspicious. The IHA titers of duplicate samples were interpreted using geometric means of titer: 1:80 or more, positive; 1:20 or less, negative; and mean titers between 1:20 to 1:80, suspicious.

2) Modified IHA 1 (Assay at 37 °C): The assay was performed with duplicate samples by the same method as the

standard IHA procedure except the plates were incubated at 37 °C over night, after addition of S-RBC.

3) Modified IHA 2 (Preincubation of sera with pig erythrocytes): Blood was collected with a 0.15% EDTA solution from six noninfected pigs. The plasma and buffy coat were removed by centrifugation and the erythrocytes were washed 3 times with PBS. The erythrocytes of the 6 pigs were pooled. Inactivated test sera (150 µl) were incubated with the pooled erythrocytes (100 µl) either at 37 °C or 4 °C for 2 hours. After incubation, the mixtures were centrifuged and 100 µl of supernatant (pig erythrocyte absorbed sera) were tested, in duplicate, by the standard IHA procedure.

Enzyme-linked immunosorbent assay (ELISA). In 96 well polystyrene ELISA plates (Immulon 2, Dynatech Laboratories Inc., Chantilly, VA), 2 µg of IHA antigen in 100 µl of 50 mM carbonate buffer (pH 8.5) was placed and incubated at 4 °C over night. The plates were washed once with saturation buffer (2% bovine serum albumin (BSA)-tris buffered saline (TBS) containing 0.5% Tween 20 (polyoxyethylene sorbitan monolaurate, pH 7.4) and blocked with 200 µl of saturation buffer for one hour. The plate was washed 3 times with saturation buffer and then incubated with 100 µl of serum, serially diluted by two fold with incubation buffer (0.5% BSA-TBS-Tween, pH 7.2) with a starting dilution of 1:4, for one hour at 25 °C. The plate was again washed 3 times with saturation buffer and incubated with horse radish peroxidase

(HRP) conjugated, affinity purified goat anti-pig IgM (100 times diluted in incubation buffer, Kirkegaard & Perry laboratories Inc., Gaithersburg, MD) for one hour at 25 °C. The plate was then washed 3 times with saturation buffer and 100 µl of 0.05 M phosphate-citrate buffer (pH 5.0) containing 0.01% Azino-bis (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co.) and 0.01% Hydrogen peroxide was added to each well. After 30 minutes of color development at 25 °C, the optical density of each well was read at 405 nm by an ELISA reader (EIA Reader Model EL 307, Bio-Tek Instruments Inc., Burlington, VT).

Electrophoresis and western blot analysis of the antigens. The Es^+ and Es^- antigens obtained by each preparation method were mixed with sample buffer (100 mM Tris (pH 6.8), 3.3% (w/v) SDS, 16.7% (v/v) glycerol, 4.2% (v/v) β-mercaptoethanol, and a few crystals of bromophenol blue) in 5 ml glass tubes and put into boiling water for 3 minutes. SDS-polyacrylamide gel electrophoresis (PAGE) was performed with these antigens using a 9% gel (Smith, 1984). The proteins in the gel were visualized using Coomassie brilliant blue stain.

Proteins were transferred to nitrocellulose membranes (NC's) electrophoretically by the method of Towbin et al. (1979). The proteins transferred to the NC's were blocked with saturation buffer (same formula as ELISA procedure) at 4 °C over night. The NC's were incubated with sera, diluted

1:100 with incubation buffer (same formula as ELISA procedure), at 25 °C for two hours and washed 3 times with the washing buffer, 0.2% BSA-PBS-0.5% Tween (pH 7.0), 10 minutes each wash. The NC's were incubated with either HRP conjugated goat anti-pig IgM (1:500 in the incubation buffer, Kirkegaard & Perry laboratories Inc.) or rabbit anti-pig IgG (1:2,000 in the incubation buffer; Sigma Chemical Co., St. Louis, MO) for another hour and washed 3 times. The NC's were finally incubated for two hours with triethanolamine buffered saline (pH 7.5) containing 0.05% 4-chloro-1-naphthol (Sigma Chemical Co.) and 0.01% hydrogen peroxide.

E. suis specific bands were determined by comparing the bands present in western blots using combinations of Es^+ and Es^- antigens, and immune and nonimmune sera. Bands detected only in the western blots using Es^+ antigens and immune sera were considered to be *E. suis* specific antigen bands.

Protein standards used for molecular weight (MW) markers (SDS-6H; Sigma Chemical Co.) consisted of bovine erythrocyte carbonic anhydrase (MW 29,000), ovalbumin (MW 45,000), bovine plasma albumin (MW 66,000), rabbit muscle phosphorylase B (MW 97,400), *Escherichia coli* β-galactosidase (MW 116,000) and rabbit muscle myosin (MW 205,000). The transferred MW standards were visualized by India ink stain (Hancock & Tsang, 1983).

^{35}S-methionine radiolabeling of E. suis. Methionine deficient Eagle's minimum essential medium (pH 7.3) was prepared by dissolving powder (for 1 liter of medium) of L-leucine, L-lysine and L-methinonine deficient Eagle's medium (Sigma Chemical Co.) containing Earle's salts and L-glutamine, in 1100 ml of distilled water, supplemented with 0.36 mM L-leucine, 0.36 mM L-lysine, non-essential amino acids (for one liter medium, Sigma Chemical Co.), 25 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.1% (w/v) glucose, 0.2% (w/v) sodium bicarbonate, 0.2 mM β-mercaptoethanol, 2.0 mM sodium pyruvate, 0.1 mM hypoxanthine, 0.016 mM thymidine and 0.2% (w/v) inosine. Infected (91% parasitemia) and noninfected blood were collected with sodium heparin solution (30 U/ml), and plasma and buffy coat were removed by centrifugation. The erythrocytes were washed with the deficient medium 3 times. The infected and uninfected erythrocytes were individually mixed with the deficient medium (5% packed cell volume), put into 60 mm Petri dishes, and incubated for 30 minutes in a 5% CO_2 incubator at 37 °C. After an initial incubation to starve the organisms for methionine, the culture dishes were supplemented with 20 μCi/ml of ^{35}S-methinonine (ICN Biomedicals, Inc., Irvine, CA) and 10% heat denatured FCS, and incubated for another 4 hours. After incubation, the medium was removed by centrifugation. The erythrocytes were washed twice with the deficient medium and suspended in the same volume of the deficient medium containing 10% FCS. The suspension was then

supplemented with 6.7% and 10% (v/v) of 40% PVP and glycerol, respectively, and stored at -80 °C. Es^+ and Es^- 100K antigens (antigen preparation Method D'spin-Es) were prepared from the frozen suspensions of infected and uninfected erythrocytes, respectively. Aliquots of the antigen preparations were removed, added to 5 ml of scintillation cocktail (High flash point cocktail, Safety-solve; Research Products International Corp., Mount Prospect, IL), and their radioactivity was measured with a liquid scintillation counter (Beta Trac; Tm Analytic, Elk Grove Village, IL). The protein concentrations of the antigen preparations were measured by Bio-Rad protein assay (Bio-Rad, Richmond, CA).

SDS-PAGE was performed with radiolabeled and normal 100K antigens in the same gel to compare the protein bands that appeared by autoradiography and western blotting. After SDS-PAGE was completed, the gel containing MW standards and radiolabeled antigens was cut, stained by Coomassie brilliant blue stain to visualize MW standards, and dried on paper using a gel drier (Bio-Rad). A direct autoradiograph was performed using Kodak X-omat RP film, 10 days exposure at 25 °C. Simultaneously, western blotting was performed with the other portion of the gel containing non-radioactive antigens, using the immune and nonimmune reference sera.

Statistical analysis. Statistical analysis was performed using the computer program StatView (version 4.0, Abacus Concepts, Berkeley, CA). The paired sign test was used for

comparing the results of the standard IHA and modified IHA's; P values less than 0.05 were considered significant.

Results

Study I. Evaluation of IHA

The effect of incubation temperature on IHA results. The effects of incubation at 37 °C (modified IHA 1), instead of 4 °C (standard IHA), are presented in Table 3-1. With incubation at 37 °C, the titers of positive and suspicious sera tended to change (either decrease or increase) although the results were not statistically significant. A significant number of negative sera exhibited an increased titer. The magnitude of titer change in most sera was no more than two fold. Notably, 3 positive sera exhibited more than a two-fold reduction from the standard IHA titer when

Table 3-1. Alteration of indirect hemagglutination assay (IHA) titer of sera incubated at 37 °C instead of 4 °C used in the standard IHA titer.

		Change in titer by incubation at 37°C		
Serum group*	Total	No. Decreased	No. Increased	No. Unchanged
Positive	14	4 (3)†	7	3
Suspicious	10	5 (2)	2	3
Negative‡	24	4	15 (1)	5

* = Serum samples were grouped by the interpretation of the standard IHA titer (incubating at 4 °C).

† = The parentheses show the number of serum samples exhibited more than two-fold change in the titer.

‡ = Significant difference (P=0.0192) in the standard titer and modified IHA 1 titer was observed by paired sign test in the negative serum group.

incubated at 37 °C. The titers of these 3 samples were 1:80, 1:80 and 1:640 in the standard IHA and 1:20, 1:10 and negative (<1:10) in the modified IHA 1, respectively.

The effect of preincubation of serum with pig erythrocytes at different temperatures. Table 3-2 describes the alteration of titers by preincubation with pooled pig erythrocytes at 4 and 37 °C (modified IHA 2). Titers tended to decrease with preincubation at either temperature. Titer reduction with preincubation at 37 °C was greater, especially in the positive serum group than with preincubation at 4 °C (37 °C - 9 of 14 vs 4 °C - 5 of 14). A significant number of samples in the positive serum group exhibited a reduction in titer with preincubation at 37 °C when compared to the standard IHA test.

IHA using Es^+ and Es^- antigen preparations. Three serum samples were collected from one pig before infection, 15 and 22 days post-infection and tested by the standard IHA using various Es^+ and Es^- IHA antigens (Table 3-3). Pre-infection serum was IHA negative regardless of the antigen preparation. IHA titers of each post infection serum were similar regardless of using Es^+ antigen preparations, P2, P3 or P4, or Es^- antigen preparations, N1 or N2, and differences were within a two fold dilution. The titers of each post-infection sera measured using the commercial antigen (P1) were higher than those measured using the prepared antigens.

Table 3-2. Alteration of indirect hemagglutination assay (IHA) titer of sera preincubated with pig erythrocytes at 4 °C and 37 °C.

		Change in titer by preincubation at 4 °C			Change in titer by preincubation at 37 °C		
Serum group*	Total	No. Decreased	No. Increased	No. Unchanged	No. Decreased	No. Increased	No. Unchanged
Positive	14	5	0	9	9 (2) †,‡	0	5
Suspicious	10	3	3	4	3 (1)	0	7
Negative	24	7	1	16	9	4 (1)	11

* = Serum samples were grouped by the interpretation of their original IHA titer as measured with the standard test (no preincubation with pig erythrocytes).

† = The parentheses show the number of serum samples that exhibited more than a two fold change in the titer.

‡ = The difference in the standard IHA titer and modified IHA 2 titer with preincubation temperature at 37 °C in the positive serum group was significant (P=0.0039).

Table 3-3. IHA titers of three serum samples using different antigen preparations.

DPI of serum sample*	Antigen preparations					
	P1†	P2‡	P3‡	P4‡	N1§	N2§
0	negative	negative	negative	negative	negative	negative
15	1:2560	1:320	1:320	1:640	1:640	1:640
22	1:1280	1:320	1:160	1:320	1:320	1:160

* = Number of days post-infection serum
† = Commercial antigen (University of Illinois)
‡ = Es^+ antigens prepared from experimentally infected animals
§ = Es^- antigens prepared from uninfected animals

ELISA using Es^+ and Es^- IHA antigen preparations. The IgM ELISA using the IHA antigens were performed to evaluate IgM binding to the antigens. The ELISA optical density (OD) values of pre- and post-infection sera of 2 pigs, using various Es^+ and Es^- IHA antigen preparations are shown in Figure 3-1. The post-infection sera showed higher OD values than the pre-infection sera, regardless of the antigen preparations, except for the ELISA using P1 antigen with the sera of pig number 2. The Es^+ and Es^- antigens showed a similar pattern of OD value changes by serum dilution, except for the P1 antigen preparation. The back ground values of the P1 antigen preparation (ELISA with pre-infection sera) were higher than those of the other antigen preparations.

Figure 3-1. Changes in optical densities by serum dilution in IgM ELISA using various *Eperythrozoon suis* positive (P1-4) and negative (N1-2) antigen preparations for indirect hemagglutination assay (IHA). A and D: The optical density (OD) of ELISA using post-infection sera of pig number 1 and 2. B and E: The ELISA OD using pre-infection serum of pig number 1 and 2. C and F: The difference of OD of pre- and post-infection sera of pig number 1 and 2, rescectively. P1 = *E. suis* positive antigen obtained from University of Illinois. P2-4 = *E. suis* positive antigens prepared from experimentally infected pigs. N1-2 = *E. suis* negative antigens prepared from uninfected pigs.

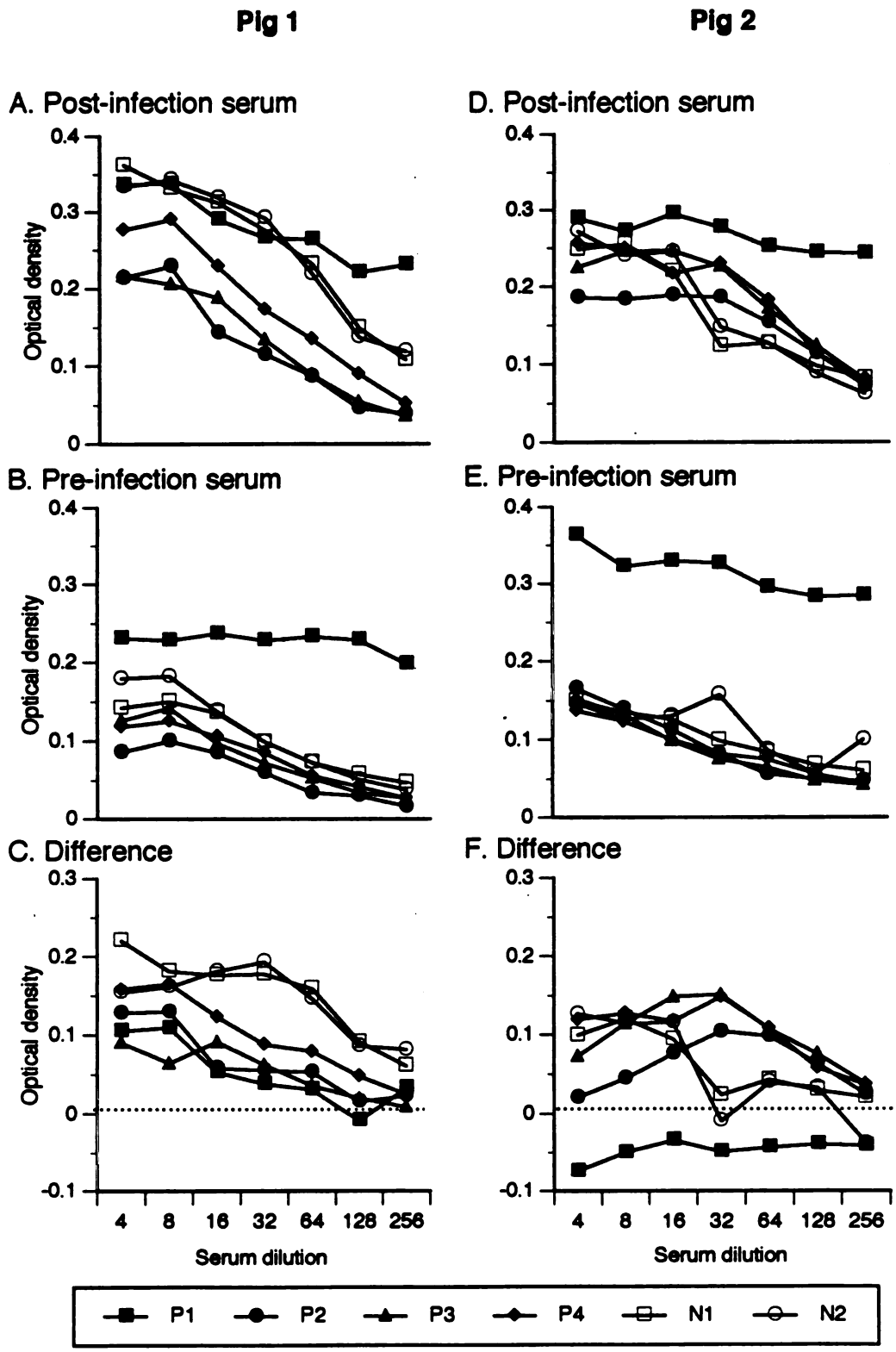
Pig 1
Pig 2
A. Post-infection serum
D. Post-infection serum
B. Pre-infection serum
E. Pre-infection serum
C. Difference
F. Difference
Optical density
0.4
0.3
0.2
0.1
0
-0.1
4
8
16
32
64
128
256
Serum dilution
P1
P2
P3
P4
N1
N2

Figure 3-1.

SDS-PAGE and western blot analysis of IHA antigens. SDS-PAGE of various IHA antigens stained by Coomassie brilliant blue are depicted in Figure 3-2. Although there were differences in band intensity, the protein band patterns in SDS-PAGE of Es^+ and Es^- antigens were nearly identical. Es^+ antigen preparation specific bands could not be clearly identified. Intense, wide bands were observed at the 80 to 85 kd area and 65 to 75 kd area in each antigen preparation. Inconsistent, lighter bands were also observed in each antigen preparation. The P1 antigen showed an additional wide band at 60 kd, which was detected as a thin band in other antigen preparations.

In IgM western blots, almost identical bands were observed in Es^+ (P2-4) and Es^- (N1-2) antigens reacted with immune sera; however, they lacked a 60 kd band observed with the P1 antigen preparation (Figure 3-3 A). The 60 kd protein band in the P1 antigen was detected as a dense wide band in blots reacted with both immune and nonimmune sera. The intensity of all bands observed was greater in the blots using the immune reference serum than those using the nonimmune reference serum. In IgG western blots, the band patterns were again similar in all antigen preparations; however, the P1 antigen had denser bands, especially the 60 kd protein band was observed strongly in the western blots with immune and nonimmune reference sera (Figure 3-3 B). Also in blots with no serum applied, the intensity of the 60 kd protein band was similar to the immune and nonimmune sera

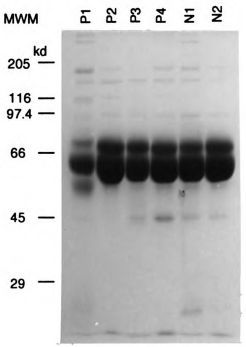

Figure 3-2. Comparison of the protein composition of various *Eperythrozoon suis* positive (Es^+) and negative (Es^-) antigen preparations for indirect hemagglutination assay (IHA) by SDS-PAGE stained by the Coomassie brilliant blue. Each lane of a 9% gel was loaded with 40 μg of reduced conditioned antigen preparation. P1 = Es^+ antigen preparation obtained from the University of Illinois, P2-4 = Es^+ antigens prepared from experimentally infected pigs, N1 and 2 = Es^- antigens prepared from uninfected pigs. MWM = Molecular weight marker.

Figure 3-3. Western blot analysis of indirect hemagglutination assay antigen preparations. A: Western blots using 40 μg of *Eperythrozoon suis* positive (Es^+) and negative (Es^-) antigens with 1:100 diluted immune and nonimmune reference sera, detected by horse raddish peroxidase (HRP) conjugated anti-pig IgM and 4-chloro-1-naphthol. B: Western blots using 40 μg of Es^+ and Es^- antigens with 1:100 diluted immune and nonimmune reference sera and without serum, detected by HRP conjugated anti-pig IgG and 4-chloro-1-naphthol. Lane P1: Es^+ antigen obtained from University of Illinois. Lanes P2-4: Es^+ antigens prepared from experimentally infected animals. Lanes N1 and 2: Es^- antigens prepared from uninfected pigs.

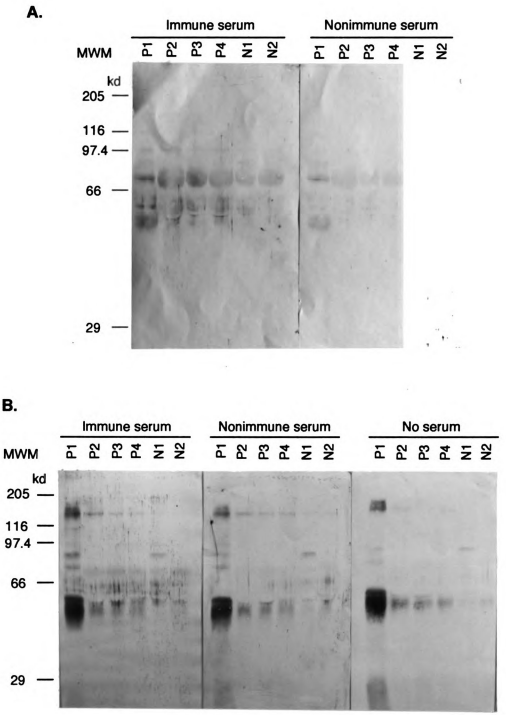
A.
Immune serum
Nonimmune serum
MWM
P1
P2
P3
P4
N1
N2
kd
205
116
97.4
66
29
B.
Immune serum
Nonimmune serum
No serum
MWM
kd
205
116
97.4
66
29

Figure 3-3.

reacted blots. As observed in the IgM blots, the band intensity was greater in the blots using immune reference serum than those using nonimmune reference serum.

In summary, *E. suis* specific bands were not readily detected in the Es^+ IHA antigen preparations either by SDS-PAGE, or IgM or IgG western blotting.

Study II. Evaluation of Antigens Prepared by Three Different Methods (Methods EDTA-Es, Plasma-Es and D'spin-Es)

Antigens prepared by Method EDTA-Es. IgG western blots using Es^+ and Es^- antigens prepared by Method EDTA-Es and immune and nonimmune reference sera are presented in Figure 3-4. Several possible *E. suis* specific antigens were detected as faint bands at 50 to 55 kd and 25 kd in the Es^+ antigen/immune serum lane. However, the predominant protein bands detected were observed both in the Es^+ and Es^- antigen preparations and were considered to be host derived proteins. Method EDTA-Es was also unacceptable because the procedure resulted in severe hemolysis during the incubation of parasitized RBC's with EDTA-PBS. Also, approximately half of the samples became gelatinous during incubation and no supernatant could be obtained from the preparation following centrifugation. Therefore, clear supernatant containing free *E. suis* could not be readily obtained.

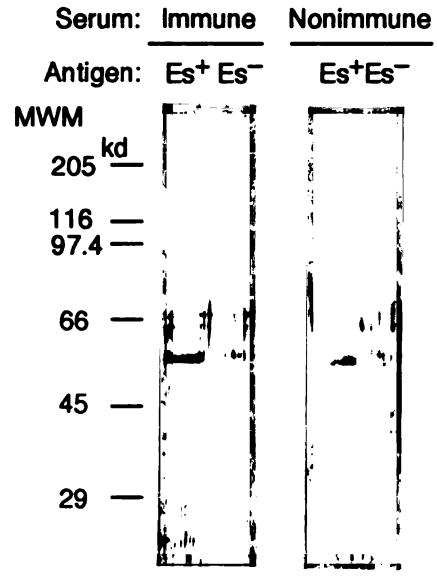

Figure 3-4. Western blot analysis of *Eperythrozoon suis* positive (Es^+) and negative (Es^-) antigens prepared by Method EDTA-Es. The blots of the Es^+ and Es^- antigens (8 μg in each lane) were incubated with the immune and nonimmune reference sera and detected by horse raddish peroxidase (HRP) conjugated anti-pig IgG and 4-chloro-1-naphthol. MWM = Molecular weight marker.

Antigens prepared by Method Plasma-Es. IgG western blotting was performed using Es^+ and Es^- antigens prepared by Method Plasma-Es-A with pre- and 2, 4 and 8 weeks post-infection sera of experimentally infected animals with *E. suis* (Figure 3-5). A small number of bands including MW 160, 54 and 26 kd antigens were observed in the western blots with the pre-infection sera. In addition, a number of bands were detected by post-infection sera, especially at 4 and 8 weeks. Intense bands were detected at MW 104, 78 and 40 kd. However, most of the bands observed in the blots using the Es^+ antigen preparation with post-infection sera were also observed in blots using Es^- antigen preparations reacted with post-infection sera. Consequently, most of the bands detected were determined to be host derived proteins. Clearly defined *E. suis* specific bands were not readily apparent except for a possible *E. suis* specific band at MW 35 kd.

Percoll separation of the antigen pellets was then attempted (Method Plasma-Es-B). Gradient centrifugation resulted in three visible layers with the Es^+ antigen pellet and one with the Es^- antigen pellet. However, clearly defined *E. suis* particles were not observed in these layers by electron microscopy (data not shown) and western blotting was not attempted.

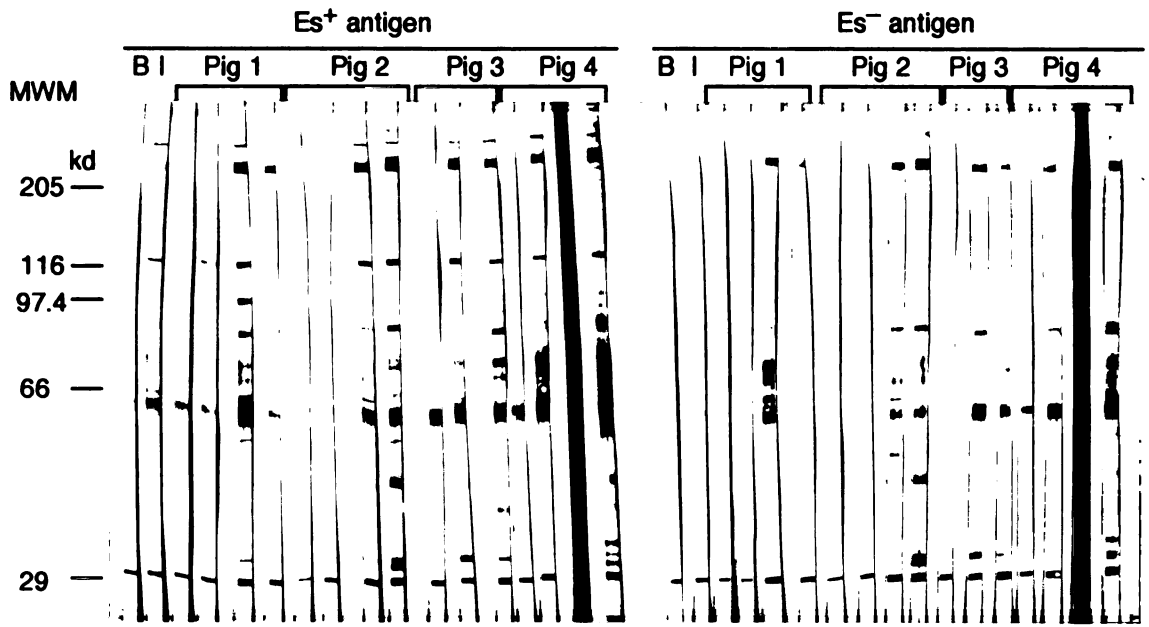

Figure 3-5. Western blot analysis of *Eperythrozoon suis* positive (Es^+) and negative (Es^-) antigens prepared by Method Plasma-Es. The blots of the Es^+ and Es^- antigen preparations were cut into strips and incubated with no serum (B), and 1:100 diluted immune reference serum (I) and 4 serum samples each from 4 experimentally infected pigs with *E. suis*. The 4 serum samples were collected before infection, and at 2, 4 and 8 weeks post infection (from left to right). The 2 weeks post infection serum was not collected from pig number 3. The antigens were detected by horse raddish peroxidase (HRP) conjugated anti-pig IgG and 4-chloro-1-naphthol. MWM = Molecular weight marker.

Antigens prepared by Method D'spin-Es. The thaw, 500, 10K and 100K antigens prepared from infected and noninfected blood were compared by IgG western blotting. A number of *E. suis* specific antigen bands, detected only in the western blots using the Es^+ antigen preparations with immune reference sera, were observed in each Es^+ antigen preparation (Figure 3-6). Strong *E. suis* specific bands were detected at MW 23, 24, 37, 47, 50, 71, 79, 81, 101 and 106 kd in Es^+ thaw, 10K and 100K antigen preparations. The 500 antigen preparation lacked some of these *E. suis* specific antigens. The 23, 24, 37, 47, 77, 79, and 106 kd antigens appeared most dense in the Es^+ 100K antigen. In addition, *E. suis* specific bands at MW 36 and 77 kd were detected strongly only in the 100K antigen preparation. A band at 90 kd was detected in both Es^+ and Es^- preparations of thaw, 500 and 10K antigen preparations. In 100K antigen preparations, however, the band at 90 kd was only detected in the Es^+ preparation (shown by arrow).

Several host derived antigens, determined by the band appearance in the Es^- antigen preparations, were also observed in all preparations. The dense host derived antigen bands were detected at MW 200 (doublet) and 26 kd. However, the 200 kd antigens were found to be less intense in the 100K antigen preparation compared to the 500 and 10K antigen preparations.

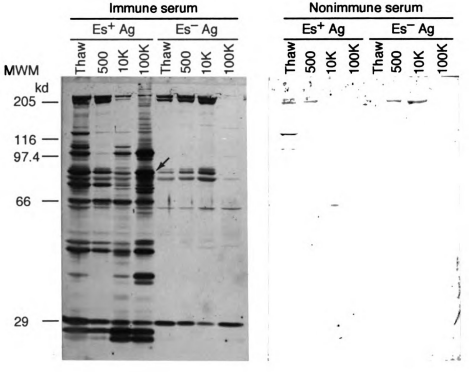

Figure 3-6. Western blot analysis of various antigens prepared by Method D'spin-Es. The blots of 40 μg of each *Eperythrozoon suis* positive (Es^+) and negative (Es^-) thaw, 500, 10K and 100K antigen preparations were incubated with the 1:100 diluted immune and nonimmune reference sera. The antigens were detected by horse radish peroxidase (HRP) conjugated rabbit anti-pig IgG and 4-chloro-1-naphthol. MWM = Molecular weight marker. An arrow shows a 90 kd antigen observed in 100K antigen preparation.

Host protein concentration appeared to be the most reduced and the greatest number of *E. suis* specific bands could be recognized in the 100K antigen preparation.

Study III. 100K Antigen Preparation

Western blots with various sera. Western blots using Es^+ and Es^- 100K antigen preparations with pre- and post-infection sera obtained from 5 pigs are shown in Figure 3-7. *E. suis* specific antigen bands readily detected in Study II were consistently observed in all western blots using the Es^+ antigen preparation with post-infection sera. Specifically, antigen bands at molecular weights of 23, 24, 37, 47, 71, 90 and 106 kd were strongest.

Western blots using antigens prepared at different stages after experimental infection. Figure 3-8 shows the western blots using different 100K antigens prepared from blood collected at 6 different times (prior to infection, during parasitemia and during remission), with the immune and nonimmune reference sera. During the first parasitemia, only the 24, 90 and 106 kd *E. suis* antigens, were detected in the antigens prepared from parasitemic blood. During the second parasitemia, other *E. suis* antigens (23, 37, 47, 71, 79 and 81 kd) were observed in addition to the 24, 90 and 106 kd *E. suis* antigens.

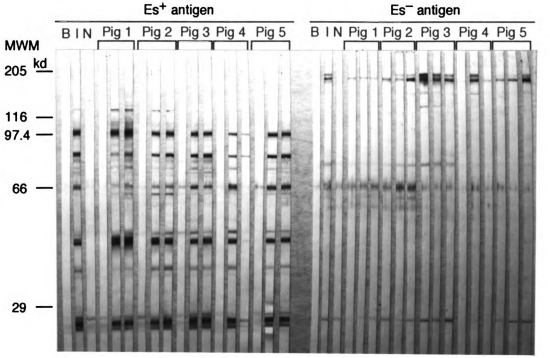

Figure 3-7. Western blot analysis of the 100K antigen using sera from 5 experimentally infected pigs with *Eperthrozoon suis*. Blots of both *E. suis* positive (Es^+) and negative (Es^-) 100K antigen preparations were incubated with no serum (B), immune reference sera (I), nonimmune reference sera (N), and three sera each from five different pigs. The three sera were collected before infection, and at 2 and 4 weeks post infection (from left to right). MWM = Molecular weight marker.

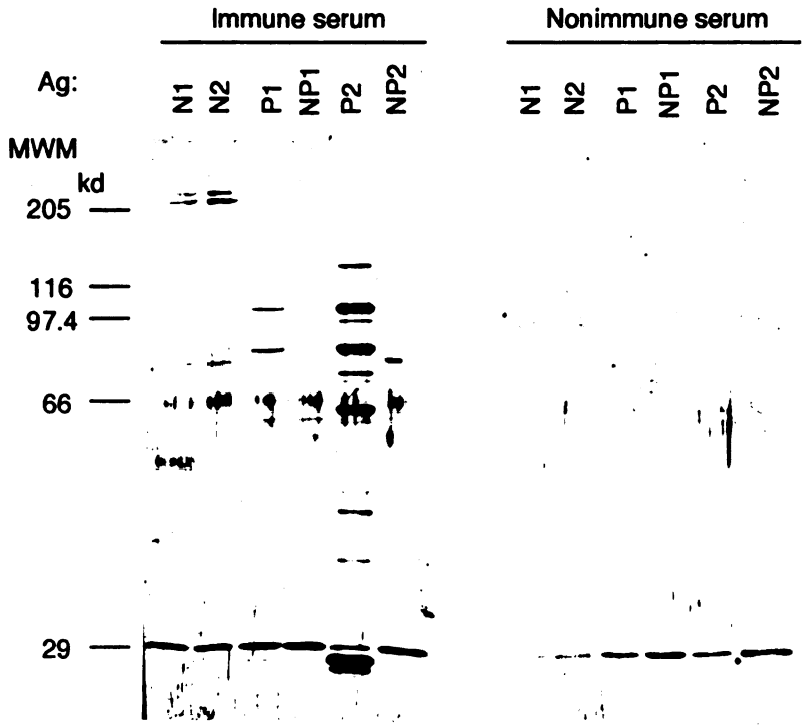

Figure 3-8. Western blot analysis of the 100K antigen prepared at the various times before or after infection. The blot of 40 μg of each antigen preparation was incubated with the 1:100 diluted immune and nonimmune reference sera and detedted with horse raddish peroxidase (HRP) conjugated rabbit anti-pig IgG and 4-chloro-1-naphthol. The antigens used were prepared from blood collected before splenectomy (N1), after splenectomy and before infection (N2), at the first parasitemic phase (P1), after the first parasitemic phase (NP1), at the second parasitemic phase (P2), and after the second parasitemic phase (NP2). MWM = Molecular weight marker.

SDS-PAGE and autoradiography using ^{35}S-methionine labeled antigen preparations. The autoradiograph of radiolabeled antigen preparations and western blots of normal antigen preparations are compared in Figure 3-9. Twenty five protein bands were detected in the radiolabeled Es^+ antigen preparation (lane 3), while only two light bands were detected in the radiolabeled Es^- antigen preparation (lane 4). The protein bands at MW 24, 47, 66, 71, 81, 90, 101 and 106 kd observed in the autoradiograph of the Es^+ antigen preparation (shown by the left pointing arrows) were also detected in the western blot of the Es^+ antigen preparation reacted with the immune reference serum (lane 1), but not in the other western blot lanes (lanes 2, 5 and 6). Therefore, the 8 bands at MW 24, 47, 66, 71, 81, 90, 101 and 106 kd were judged to be *E. suis* derived antigens. In addition, a band was observed at MW 37 kd in the autoradiograph of the Es^+ antigen preparation (shown by the right pointing arrow). A similar band was not detected in the western blots of this experiment. However, a 37 kd *E. suis* specific antigen was consistently observed in previous western blot experiments. Therefore, the band observed at MW 37 kd in the autoradiograph was also judged to be *E. suis* derived.

Western blots with different post-infection sera of the same pigs. For this experiment, eight *E. suis* specific antigens were selected, according to their appearance in the autoradiograph of radiolabeled antigen preparations and

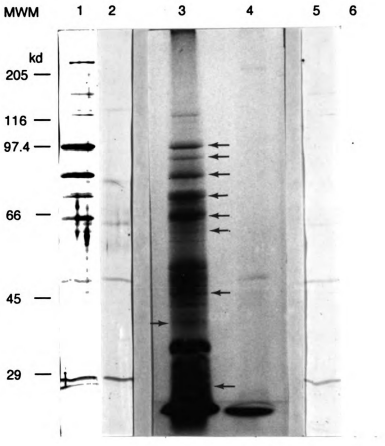

Figure 3-9. Comparison of the autoradiograph of radiolabeled 100K antigen preparations and western blots of the 100K antigen preparations. Lanes 1 and 2 show the western blots of the *Eperythrozoon suis* positive (Es^+) and negative (Es^-) antigen preparations (50 μg each) incubated with the immune reference sera. Lanes 3 and 4 show the autoradiograph of the sulfur 35 labeled Es^+ and Es^- antigen preparations (30 μg each). Lanes 5 and 6 show the western blots of the Es^+ and Es^- antigen preparations (50 μg each) incubated with the nonimmune reference sera. MWM = Molecular weight marker. Left arrows show 101, 101, 90, 81, 71, 66, 47 and 24 kd proteins. Right arrow shows 37 kd protein.

various western blots previously described. The selected bands were recognized at MW 24, 37, 47, 71, 81, 90, 101 and 106 kd. IgG western blotting was performed with the Es^+ and Es^- 100K antigen preparations using sera obtained weekly from 4 experimentally infected animals up to 21 weeks post-infection (WPI). Table 3-4 summarizes the band detection in western blots using the Es^+ and Es^- 100K antigen preparations reacted with various pre- and post-infection sera. All *E. suis* specific antigens (observed only in the Es^+ antigen preparation) were detected with all post-infection sera except in those instances reported in Table 3-4. In addition, the pre-infection sera of 2 pigs recognized the 71 kd antigen. In the Es^- antigen preparation, bands were observed at MW 71 and 90 kd, the same position as the *E. suis* specific antigens. The 71 kd antigen in the Es^- antigen preparation appeared as a rough broad band.

Western blots using post-infection sera collected weekly from one pig are shown in Figure 3-10. All 8 *E. suis* specific bands were detected as intense bands by post-infection sera collected at either 1 or 2 WPI through 15 WPI except for the 14 WPI serum. In addition to the 8 *E. suis* antigens, several other antigen bands (shown by arrows) were recognized only by sera obtained during the late stage of infection. Late appearing antigen bands were also observed in the other 3 pigs; however, antigen sizes and time recognized varied among the 4 pigs.

Table 3-4. *Eperythozoon suis* specific bands detected in the western blots using 100K *E. suis* positive (Es^+) and negative (Es^-) antigen preparations with various pre- and post-infection sera from 4 pigs.

MW of the band	Detection in Es^+ antigen preparation	Detection in Es^- antigen preparation
24	Detected by all sera from 1-2 up to 21 WPI, except for 14 WPI sera from one pig	Not detected
37	Detected by all sera from 2-3 up to 21 WPI, except for several post-infection sera from 3 pigs	Not detected
47	Detected by all sera from 1-2 up to 21 WPI, except for 14 WPI sera from one pig	Not detected
71	Detected in pre-infection sera of 2 pigs, and all 4 pigs from 1-2 up to 21 WPI, except 14 WPI sera from one pig	Detected by pre and post-infection sera of 3 pigs*
81	Detected by all sera from 2-3 up to 21 WPI, except for 14 WPI sera from one pig	Not detected
90	Detected by all sera from 1-3 up to 21 WPI, except for 14 WPI sera from one pig	Detected by 2-3 up to 16 WPI sera of 3 pigs
101	Detected by all sera from 2-3 up to 21 WPI, except for 14 WPI sera from one pig	Not detected
106	Detected by all sera from 1-2 up to 21 WPI, except for 14 WPI sera from one pig	Not detected

* = Observed as rough broad bands

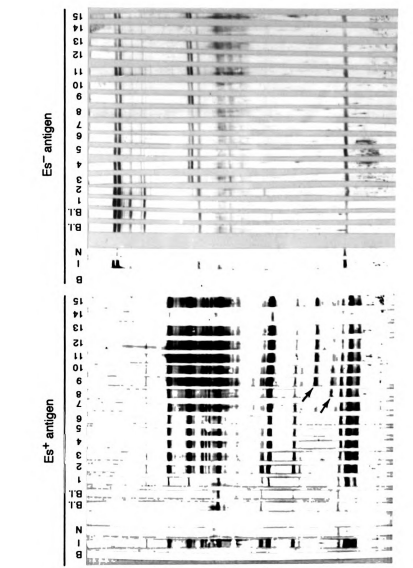

Figure 3-10. Western blot analysis of 100K antigen preparation using sera collected weekly from an experimentally infected pig. The blots of the *Eperythrozoon suis* positive (Es^+) and negative (Es^-) antigen preparations were incubated with no serum (B), the immune reference serum (I), the nonimmune reference serum (N), sera collected before infection (B.I.), and sera collected at 1 to 15 weeks post infection (shown by number of weeks post infection). Arrows show antigens recognized only by sera collected during late infection. MWM = Molecular weight marker.

Discussion

The inclusion of host erythrocyte proteins in antigen preparations has been a common cause of false positive (poor specificity) results in immunoassays with various hemoparasitic diseases such as anaplasmosis (Barbet et al., 1983; Budden & Dimopoullos, 1977; Palmer et al., 1986) and babesiosis (Callow & Dalgliesh, 1982; Goodger et al., 1985). One possible reason for this poor specificity is the reaction of antigen impurities with antibodies in sera (Goodger & Mahoney, 1974). Anti-erythrocytic antibodies in the form of cold agglutinins have been observed in hemoparasitic infections such as eperythrozoonosis, haemobartonellosis, anaplasmosis, malaria and babesiosis and are associated with anemia (Bellamy et al., 1978; Oki & Miura, 1970; Schroeder & Ristic, 1965; Soni & Cox, 1974; Soni & Cox, 1975; Thoongsuwan & Cox, 1973; Zachary & Smith, 1985; Zulty & Kociba, 1990). A majority of these cold agglutinins are IgM antibodies directed at glycoproteins (mainly I antigen in humans) located on the erythrocyte membrane (Lau & Rosse, 1975; Pruzanski & Shumak, 1977a; Pruzanski & Shumak, 1977b). In addition to cold agglutinins, isoerythrocyte antibodies have been frequently observed in experimental infection or vaccination systems using infected blood as an inoculum (Barry et al., 1986; Dimmock, 1973; Duzgun et al., 1988). The role of isoerythrocyte antibodies transferred via colostrum from dams vaccinated with lyophilized *A. marginale*

infected erythrocytes has been proposed in neonatal isoerythrolysis of calves (Ristic, 1976).

Study I examined the role of cold agglutinins in IHA by comparing the standard IHA to several modified procedures. With the modified IHA 1 (incubation at 37 °C), 77% of the sera (37 of 48 tested) showed either an increase or decrease in titer compared to their respective standard IHA titers (Table 3-1). A significant number of samples in the negative serum group showed an increased titer with the modified IHA 1. However, the magnitude of the titer change of most samples was no more than two-fold. More than two-fold titer changes were observed mainly in the positive and suspicious sera, which exhibited marked decreases in titers with the modified IHA 1. The decrease in titers observed with positive sera indicates an active role of cold temperature reacting antibodies in IHA and demonstrates that the incubation temperature used in the standard IHA (4 °C) needs to be maintained to provide consistent results.

The modified IHA 2 (preincubation of sera with normal pig erythrocytes) was designed to evaluate the role of autoantibodies to erythrocyte antigens in IHA. Two different temperatures were used for preincubation of sera in order to evaluate the temperature dependency of the autoantibodies. A significant number of positive sera (9 of 14) exhibited a decreased titer with the modified IHA 2, preincubation at 37 °C, compared to their respective standard IHA titers. In addition, 5 of 14 positive sera showed a decreased titer with

the modified IHA 2 with preincubation of sera at 4 °C (P value in the paired sign test was 0.0625). These results indicated that the IHA titers of positive sera decreased with preincubation of sera with pig erythrocytes regardless of the preincubation temperature and suggested that temperature independent autoantibodies also play an active role in IHA.

The role of autoantibodies in the IHA was further evaluated in subsequent experiments using Es^+ and Es^- antigen preparations. The observation that few differences were found in IHA and in IgM ELISA when using Es^+ (P2-4) or Es^- (N1 and N2) antigen preparations indicated that the main antibodies detected in IHA and ELISA were autoantibodies to erythrocyte antigens. This finding was further confirmed by failure to detect *E. suis* specific antigens by western blot analysis of the Es^+ and Es^- IHA antigens.

In the western blot procedure, antigen preparations were boiled in SDS containing buffer. Consequently, the proteins were presumably denatured and may have lost important conformational epitopes. Previously, Shankarappa and Dutta (1989) could not demonstrate protein specificity with a monoclonal antibody to *Ehrlichia risticii* by western blotting and they concluded that heat denaturation sufficiently altered the specific epitope, making it nonreactive with the monoclonal antibody. The presence of conformational antigens in the antigen preparations could not be evaluated by western blot analysis. However, antigens with altered conformation could be potentially detected as differences in protein

composition in SDS-PAGE analysis between the Es^+ and Es^- antigen preparations. In this study, no bands were detected specifically in the Es^+ antigen preparations compared to Es^- antigen preparation by SDS-PAGE analysis. This finding indicates that the Es^+ antigen preparations contain insufficient *E. suis* specific antigens to be detected by SDS-PAGE, and that the majority of proteins and antigens are host derived.

Interestingly, the P1 antigen (obtained from the University of Illinois) contained an exceptionally high level of 60 kd protein. The 60 kd band in the P1 antigen was observed with equal intensity in IgG western blots reacted with immune and nonimmune sera, and also in western blots reacted with no serum. The strong band appearance in western blots with no serum indicated that the 60 kd protein reacted with anti-pig IgG, thus suggesting that the 60 kd protein was a part of IgG, possibly the heavy chain portion based on the protein molecular weight. This observation alternatively suggests a possibility of the interference in test results by the IgG (either *E. suis* specific antibodies or erythrocyte autoantibodies induced by *E. suis* infection) inclusion in IHA antigen preparation. For example, hemagglutination could occur due to bridging *E. suis* specific IgG present in the IHA antigen preparation by *E. suis* bodies present free in serum obtained from parasitemic pigs, which could increase titer levels and test sensitivity, complicating interpretation of true *E. suis* IgM levels/seroconversion. Alternatively,

hemagglutination could result from bridging of erythrocyte autoantibodies present in IHA antigen preparation by erythrocyte antigens present in serum, possibly reducing test specificity.

The 60 kd band was also observed in IgM western blot using the P1 antigen with immune and nonimmune sera, although the band intensity was reduced. This observation could be interpreted as a cross reaction between anti-pig IgM and the 60 kd protein. This cross reaction could also explain the high background values observed in the IgM ELISA using the P1 antigen.

In the IHA experiment using various antigen preparations, the P1 antigen yielded 2-3 fold higher titers compared to other antigens. The differences observed in SDS-PAGE between the P1 antigen and other antigens may explain the titer differences. If one assumes that the 60 kd protein is IgG heavy chain, this protein is not "a functional protein" for the agglutination reaction. Sheep erythrocytes were coated with the same amount of each IHA antigen preparation based on the total weight of proteins. Since the P1 antigen preparation contains an exceptional amount of the 60 kd protein, the relative amount of "functional proteins" in the P1 antigen used for coating sheep erythrocytes would be lower than the relative amount of "functional proteins" in other antigen preparations (P2, P3, P4, N1 and N2). If it is assumed that the amount of "functional proteins" in the P1 antigen was optimal for the agglutination reaction, and that

the amounts of "functional proteins" in the other preparations were greater but sub-optimal for the agglutination reaction, IHA using P1 antigen preparation may have yielded higher titers than IHA using other antigen preparations.

Isolation of *Eperythrozoon* bodies has been attempted with *E. ovis* and *E. suis*. Lang et al. (1986) described successful separation of *E. ovis* bodies from hemolysates. Following this method, Hall et al. (1988) failed to completely separate *E. suis* bodies from erythrocyte antigens by lysing erythrocytes. Alternatively, they used EDTA to release the organisms from erythrocytes, and then separated the free organisms from erythrocytes by low speed centrifugation. The organisms were then concentrated by high speed centrifugation, yielding a high concentration of *E. suis* bodies. In the first experiment of Study II (Method EDTA-Es), the method of Hall et al. (1988) was applied to separate *E. suis* and found to be inadequate for purifying the organisms. In addition, severe hemolysis and the formation of a gelatinous precipitate of parasites and erythrocytes occurred during the incubation of the parasitized RBC's with EDTA-PBS, making this method unacceptable for separating *E. suis* from blood components. The cause of the severe hemolysis may be explained by an increased fragility of the erythrocytes due to *E. suis* infection. Increased fragility of infected erythrocytes has also been observed in babesiosis (Wright, 1973) and haemobartonellosis (Maede, 1980). In

Haemobartonella felis infected erythrocytes, increased osmotic fragility was found to be related to decreased erythrocyte lipids concentration (Maede, 1980). The cause of the gelatinous precipitation of infected erythrocytes and the EDTA-PBS mixture during the incubation was not determined although it may be related to activated platelets that remained after the washing step. The activation of platelets during parasitemia has been observed in *E. suis* infected pigs (Plank & Heinritzi, 1990; Zachary & Smith, 1985) and *E. ovis* infected sheep (Overås, 1969).

In the second experiment of Study II (Method Plasma-Es), an attempt was made to isolate *E. suis* free in plasma. However, the antigen preparation contained excessive host blood components, mostly platelets as determined by light microscopy, and clear separation of *E. suis* was not achieved. The source of the various bands observed in the western blots was not clear, but some of them may have been derived from platelet receptor proteins and also IgG bound to platelets. In the subsequent Percoll separation procedure, *E. suis* bodies could not be detected by electron microscopy in the visible layers formed, indicating Method Plasma-Es was not sufficient for separating *E. suis* free in plasma from plasma components.

In the final experiment of Study II (Method D'spin-Es), *E. suis* isolation was attempted from infected erythrocyte lysates. Each pellet of the differential centrifugation procedure contained various *E. suis* specific antigens. The

observation that the 100K antigen preparation contained the least amount of host antigens and the most variety of *E. suis* specific antigens indicated that differential centrifugation concentrated *E. suis* antigens effectively.

Method D'spin-Es provided another advantage that frozen infected erythrocytes could be used. In Methods EDTA-Es and Plasma-Es, parasitemic pigs were required as fresh infected blood was used. With Method D'spin-Es, infected erythrocytes could be stored frozen and the antigen could be prepared or the antigen preparation method could be modified at any time.

In Study III, the 100K antigen preparation was further evaluated for *E. suis* specificity by various methods. Western blots using sera from 5 pigs revealed several *E. suis* specific antigens at the same molecular weights as observed in Study II, further confirming the *E. suis* specificity of the 100K antigen preparation. However, there was a possibility that some of the observed *E. suis* specific antigens may have been erythrocyte antigens altered by *E. suis* infection. These altered erythrocyte antigens could be retained in the antigen preparation prepared from the post parasitemic blood in the second experiment of Study III, because it has been observed that erythrocyte membrane alteration was retained after *E. suis* was eliminated from the blood by tetracycline treatment (Zachary & Basgall, 1985). In the present experiment, *E. suis* specific antigens were detected only in antigen preparations from parasitemic blood and not from blood collected during remission, indicating

that the observed antigens were *E. suis* derived proteins and not proteins derived from erythrocytes that may have been altered by *E. suis* infection. In the same experiment, the numbers of *E. suis* specific antigens observed in the 100K antigen preparations from the 1st and the 2nd parasitemic phase blood were different. This was probably due to the difference in concentrations of *E. suis* bodies in the 2 antigen preparations. The degree of parasitemia at the 1st and 2nd parasitemic phase were 72 and 96%, respectively. The first parasitemic phase antigen was the only antigen prepared from less than 90% parasitemic blood in all of the experiments. Considering the differences in the number of *E. suis* specific antigens detected in the 1st and 2nd parasitemic phase antigens, it was assumed that preparing the antigen from blood with a lower parasitemia resulted in lower concentration of *E. suis* specific antigens.

Antigens prepared from infected erythrocyte lysates have been developed in various hemoparasites infection. In swine eperythrozoonosis, whole lysate of infected erythrocytes have been used in the complement fixation test (Splitter, 1958), and ELISA (Schuller et al., 1990). Hsu et al. (1992) developed an *E. suis* antigen prepared from infected erythrocyte lysate with 2 purification steps. In their procedure, lysed erythrocytes were centrifuged at 1,000g and the supernatant was discarded. The pellet was suspended in PBS and frozen. The frozen suspension was then thawed and centrifuged at 11,000g. The resultant supernatant was used

for the antigen in ELISA. In ovine and bovine eperythrozoonosis, semi-purified organisms from infected erythrocyte lysates have been used for complement fixation, ELISA and passive hemagglutination tests (Daddow, 1977; Finerty et al., 1969; Kawazu et al., 1990; Lang et al., 1987). In anaplasmosis, purification of *A. marginale* from erythrocyte lysates has been attempted using enzymatic digestion of erythrocyte components (Hart et al., 1981) and density gradient centrifugation (McCorkle-Shirley et al., 1985). Palmer and McGuire (1984) purified *A. marginale* by erythrocyte disruption with minimal sonication and centrifugation at a force not sufficient to sediment erythrocyte stroma. However, none of these antigen preparation methods could guarantee complete absence of host proteins or complete integrity of the parasite. Therefore, analysis of such preparations would not reliably characterize the protein structure of the organism.

In the erythrocyte culture system used for *A. marginale*, it has been demonstrated that infected erythrocytes incorporated radiolabeled amino acids but uninfected erythrocytes did not (Davis et al., 1978). *A. marginale* protein structure has been analyzed under the complete absence of host erythrocyte protein contamination, using organisms radiolabeled by metabolic incorporation of ^{35}S-methionine (Barbet et al., 1983). A methionine requirement was also demonstrated in malaria parasites (Jensen, 1983). To identify *E. suis* protein structures, radiolabeling of *E.*

suis by ^{35}S-methionine was accomplished using the erythrocyte culture system developed in a previous study (Chapter 2). The 100K antigen preparation, using ^{35}S-methionine labeled *E. suis*, demonstrated 25 distinct protein bands. Only 2 faint bands were observed in the antigen preparation from uninfected erythrocyte culture. Several of the radiolabeled *E. suis* protein bands showed the same molecular weight as the *E. suis* specific bands observed in the western blot experiments, further confirming the *E. suis* specific bands observed in the western blots were *E. suis* derived. However, some of the *E. suis* specific bands observed in the western blot experiments were not detected by autoradiography. Since radiolabeling was performed for only 4 hours, it could be assumed that only proteins with sufficiently rapid turnover were radiolabeled effectively and proteins with a relative slow turnover were not.

Alterations in erythrocyte membrane proteins due to infection may be another potential reason for the inconsistencies of *E. suis* specific antigens between the autoradiographs and western blots. In bovine anaplasmosis, it has been observed that a high molecular weight glycoprotein observed in normal bovine red cells was not present in infected red cells. Instead, four new lower molecular weight glycoproteins were found in infected erythrocytes (Nordelo & Ysern-Caldentey, 1982). These new glycoproteins were suggested to be the result of structural modifications of erythrocyte proteins due to *Anaplasma*

infection. Goff et al. (1986) found that reactions to lectins by bovine erythrocytes were altered by *A. marginale* and *E. wenyoni* infection. They suggested that additional lectin binding sites were exposed by alteration of erythrocyte membrane proteins by rickettsial infection. In addition, alterations of erythrocyte proteins have been postulated in *Plasmodium* infection as a result of changes in the relative intensity of the protein bands in SDS-PAGE, phosphorylation patterns, insertion of a new polypeptide, and recognition of the erythrocyte proteins by monoclonal antibodies (Chaimanee & Yuthavong, 1979; Crandall & Sherman, 1991; Yuthavong et al., 1979). Surface proteins and glycoproteins have been observed to be altered during infection with *Babesia bovis* (Howard et al., 1980). In swine eperythrozoonosis, alteration of erythrocyte membranes has been observed by electron microscopy and shown to persist even after the parasites were eliminated by tetracycline treatment (Zachary & Basgall, 1985). Zachary and Smith (1985) suggested that recognition of altered erythrocyte antigens as foreign antigen by the host's immune system may cause autoantibody production in swine eperythrozoonosis. Therefore, it could be assumed that some of the apparent *E. suis* specific antigens may be altered erythrocyte proteins caused by *E. suis* infection.

The source of the two faint bands observed in the autoradiograph of the antigen preparation from the uninfected erythrocyte culture material was not clear. Since mature

erythrocytes do not synthesize any proteins, the proteins of mature erythrocytes could not be radiolabeled by incorporating ^{35}S-methionine. Possible sources of the bands may be a small number of metabolically active leukocytes left over after buffy coat removal, or the presence of immature erythrocytes in the culture system.

Based on IgG western blotting and autoradiography results, eight protein bands with MW 24, 37, 47, 71, 81, 90, 101 and 106 kd, were selected as the most reliable *E. suis* specific bands. The final experiment was designed to evaluate the time and duration that the sera of experimentally infected pigs recognized these eight antigens after *E. suis* inoculation. Nearly all of the eight antigen bands were consistently recognized in western blots using sera collected from 2 to 21 weeks post infection from all four pigs evaluated, indicating that the selected antigens were highly antigenic. One week post-infection sera of all four pigs failed to recognize the antigens of MW 37, 81 and 101 kd and some sera obtained at one week post infection failed to recognize other antigens as well. Therefore, the IgG immune response occurred within 1 to 2 weeks and lasted up to 21 weeks post infection.

However, sera obtained 2 to 21 weeks post infection could not always recognize all of the eight selected antigens. Since *E. suis* is an extracellular parasite and exposed to immunoglobulins in the plasma, it could be expected that the quantity of *E. suis* specific IgG in the

plasma, collected at the parasitemic phase, may be significantly reduced by binding to organisms present in the blood. The inability of all post-infection sera to recognize all antigens may be related to the fact that some of the sera were collected during parasitemia. This might partly explain why some of the 1 week post-infection sera failed to recognize the antigens, besides the time lag between infection and the initiation of a measurable IgG immune response. Three of the four 1 week post infection sera were collected during a parasitemic (>85%) period. On the other hand, some of the sera failed to recognize the antigens even when the sera were collected during remission.

Among the 8 selected proteins, the 90 kd protein was of specific interest. This protein was detected as an intense band in the western blots using the Es^+ 100K antigen preparation with the immune reference serum, and by autoradiography. The band appearance in the autoradiograph indicated that the 90 kd protein was derived from *E. suis*. However, in Study II, a similar band was observed at the same location (90 kd) in the Es^- preparations of thaw, 500 and 10K antigen. This band, presumed to be host derived 90 kd protein, was not detected in the Es^- 100K antigen indicating that the host derived 90 kd protein was eliminated in the 100K antigen preparation. However, in the western blots performed in Study III, the host derived 90 kd band was occasionally observed in the Es^- 100K antigen, indicating the band could not always be eliminated by differential

centrifugation. These observations suggested that the Es^+ antigen preparation could contain two proteins, both having similar molecular weight of about 90 kd, one derived from *E. suis* and one from the host. Therefore, the 90 kd antigen band was not considered to be an appropriate band for evaluating *E. suis* specificity.

The 71 kd protein was recognized by the pre-infection sera of 2 pigs. This may indicate that the 2 pigs were already infected with *E. suis* prior to inoculation or had persisting colostral antibodies. Faint broad bands were observed in the Es^- antigen preparation around the 70 kd region. These bands could be confused with the 71 kd protein; although they were not sharp, and might be a reflection of nonspecific binding of negatively charged IgG to positively charged proteins located around the 70 kd region.

Interestingly, various antigens were recognized only by late infection stage sera. The cause of this observation was not elucidated in this study but indicates that the recognition of some antigens by the host's humoral immune system was delayed. This may be related to parasite evasion mechanisms from the host's immune system. Parasites can develop escape mechanisms to avoid the destructive effects of the immunologically hostile host. Cohen (1982) summarized various immune evasion mechanisms of parasites including anatomical seclusion of parasites, modification of parasite antigens, and modification of host immune responsiveness.

The immune evasion mechanisms of *Eperythrozoon* in general are poorly understood. In experimentally infected, parasitemic pigs, lymphocytes blastogenesis induced by lipopolysaccharide was increased (Zachary & Smith, 1985). Hyperglobulinemia was also observed during parasitemia. These findings indicate that polyclonal B cell activation took place during parasitemia. Polyclonal B cell activation is an immune evasion mechanism developed by many parasites, leading to misdirected responses by the host's immune system, resulting in generalized immune suppression (Cohen, 1982). It has been commonly observed that polyclonal B cell activation induces autoantibody production, consisted with one of the characteristics of swine eperythrozoonosis. As an alternative consideration, antigen mimicry of the parasite antigens to host antigens has been discussed as a cause of autoantibody induction in rickettsial and bacterial infections (Ristic, 1976; Steere, 1989). This mechanism should be considered as a potential immune evasion mechanism developed by *E. suis*, resulting in the host's immune system failure to recognize certain *E. suis* antigens as foreign.

Surface antigens continually exposed to the host might be considered as "evolutionary antigens" by which the parasite has responded in various ways to the host response and developed various immune evasion mechanisms (Lloyd & Soulsby, 1988). For parasite survival in the host, the functional antigens of the parasite, which induce protective immune responses, need to escape from host immune system

attack. On the other hand, antigens non-functional for development of host resistance could remain susceptible to host attack. Therefore, the observed antigens recognized only by sera collected during late infection could be considered as functional antigens of *E. suis*.

This study analyzed *E. suis* antigens by western blotting. The study elucidated a lack of specificity of IHA, for detecting *E. suis* specific antibodies. Therefore, the development of improved serodiagnostic methods is a critical requirement to gain a better understanding of the disease. The 100K antigen preparation developed in the present study could provide a vital component for developing more accurate serodiagnostic methods.

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CHAPTER 4

DEVELOPMENT OF IMPROVED SERODIAGNOSTIC TESTS FOR SWINE EPERYTHROZOONOSIS

Abstract

Enzyme-linked immunosorbent assays (ELISA) using horse radish peroxidase (HRP) conjugated rabbit anti-pig IgG (IgG-ELISA) and HRP conjugated protein A (ProA-ELISA), or a dot blot (DB) using ^{125}I-protein A were performed on sera obtained from experimentally infected pigs and naturally exposed pigs (field sera) using Es^+ (*E. suis* positive) and Es^- (*E. suis* negative) 100K antigen preparations described in Chapter 3. Serum antibody levels were determined by two methods: 1) actual optical density (OD) in ELISA or percent binding of protein A in DB, using Es^+ antigen only (CrudeEs-Test); or 2) adjusted OD or percent binding calculated by subtracting the value obtained with Es^- antigen from Es^+ antigen value (NetEs-Test). The ELISA and DB results were compared to the indirect hemagglutination assay (IHA). The specificity of the rabbit anti-pig IgG and protein A reagents for detecting immunoglobulins bound to antigens were compared by western blotting and no differences in the specificities of the two reagents were found as the band patterns were identical. By 2 weeks post-infection (WPI), most sera were positive by ELISA and DB as determined by CrudeEs-Test or NetEs-Test, and by IHA. However, IHA failed to detect antibodies in all of the 1 WPI sera, while the ProA- and IgG-ELISA's and the DB detected antibodies in 71, 63 and 88% of the 1 WPI sera by CrudeEs-Test, and 57, 75 and 88% by NetEs-Test, respectively. The time course of IHA titer development following

experimental infection did not correlate with the development of *E. suis* specific antibodies detected by the other assays, using CrudeEs-Test or NetEs-Test. However, IHA titers did correlate with results obtained with the ELISA's and DB using the Es^{-} antigen, indicating that the IHA titer was not specific for *E. suis* antibodies. The IHA was detecting erythrocytic autoantibodies induced by *E. suis* infection. The results obtained with the field sera varied considerably among the tests. For example, 91% of sera were positive by IgG-ELISA CrudeEs-Test, while only 9% of sera were positive by DB NetEs-Test. However, the positive or negative results obtained by CrudeEs-Test or NetEs-Test were significantly associated between the two ELISA's and the DB, but not between the IHA and the other tests. The detection of 37, 47, 71, 81 and 101 kd antigens by field sera in IgG western blots were closely associated with the results of the two ELISA's and the DB and the NetEs-Test results with the two ELISA's were significantly associated with the detection of all 5 of the antigens. However, there was no association between IHA results and the appearance of *E. suis* specific antigen bands in the western blots. It was concluded that NetEs-Test using either ELISA or DB could provide better serodiagnostic assays for swine eperythrozoonosis compared to the standard IHA.

Introduction

Swine eperythrozoonosis is caused by the rickettsial organism, *Eperythrozoon suis*. The disease is characterized by acute ictero-anemia and is associated with poor weight gain and reproductive failure (Henry, 1979). Swine eperythrozoonosis has been reported world wide including North and South America, Africa, Europe and Asia (Henry, 1979; Hsu et al., 1990; Sisk et al., 1980; Smith, 1977; Smith, 1981).

The diagnosis of swine eperythrozoonosis has been based on herd and individual animal histories describing icteroanemia and on the demonstration of *Eperythrozoon* bodies in blood smears. Detection of the organism provides definite diagnosis; however, artifacts in blood smears have been difficult to distinguish from *E. suis* bodies in many cases (Henry, 1979). Moreover, most *E. suis* infections are subclinical without detectable parasitemia.

Serodiagnostic assays such as the complement fixation test, indirect hemagglutination assay (IHA) and enzyme-linked immunosorbent assay (ELISA) have been developed for detecting low level infections not detected by traditional blood smears (Hsu et al., 1992; Schuller et al., 1990; Smith & Rahn, 1975; Splitter, 1958). The antigens used in these assays were crude preparations from parasitemic plasma or the lysate of infected erythrocytes and thus contained host plasma and erythrocyte antigens. In swine eperythrozoonosis,

autoantibodies against erythrocytes have been postulated to play an important role in anemia development (Hoffmann et al., 1981), in the occurrence of agglutination of infected blood especially at cold temperature, and increased sedimentation rate of infected blood (Doyle, 1932; Robb, 1943; Splitter & Williamson, 1950). The occurrence of anti-erythrocyte antibodies in *E. suis* infected pigs has been shown by positive Coomb's tests (Hoffmann et al., 1981). Considering the presence of host proteins in the antigen preparations used in the serodiagnostic assays for swine eperythrozoonosis, test results may be greatly influenced by immune complex formation between host proteins and the autoantibodies induced by *E. suis* infection. As reported in Chapter 3, immunoblot analysis of the antigen preparation used in the indirect hemagglutination assay (IHA), a widely used serodiagnostic technique for *E. suis* infection, indicated that antibodies detected by IHA are mainly directed at host derived proteins. The following studies were conducted to develop improved serodiagnostic assays for swine eperythrozoonosis.

Materials and Methods

Organism. *E. suis* was initially isolated from an inappetant boar housed at the Michigan State University Swine Research Center. A diagnostic CBC revealed that the boar was parasitemic and anemic. The *E. suis* isolate has been maintained by passage in splenectomized pigs. Infected blood also has been cryopreserved by adding 6.7% and 10% (v/v) of 40% polyvinyl pyrrolidone (PVP) and glycerol, respectively, and freezing in liquid nitrogen.

Animals and experimental infection. Cross bred pigs were 6- to 13-weeks-old at the time of inoculation with *E. suis*. All animals were splenectomized using standard surgical techniques 1 to 2 weeks prior to inoculation and determined to be *E. suis* noninfected by no evidence of any temperature elevation or development of parasitemia before inoculation. Ten milliliter of frozen infected blood was thawed in a 37 °C water bath and inoculated intravenously and/or intramuscularly into the splenectomized pigs. Clinical signs and rectal temperatures were assessed daily for monitoring disease development. When the rectal temperature exceeded 40 °C, blood was collected in a vacutainer tube (Beckton Dickinson Vacutainer Systems, Rutherford, NJ) containing ethylenediamine tetraacetic acid (EDTA), and examined for parasitemia by Wright's stained thin smear. When the febrile phase lasted for more than 3 days or if an animal exhibited

severe depression or anemia (less than 20% of packed cell volume), intramuscular treatment with oxytetracycline (20 mg/kg body weight; Liquamycin LA 200, Pfizer, New York, NY) was initiated to avoid mortality.

Serum samples. Serum samples were obtained from 8 experimentally infected animals prior to inoculation with *E. suis* and weekly for the first 4 weeks post-infection (WPI) and every other week from 4 to 12 WPI. A total of 140 serum samples obtained from naturally exposed pigs (field sera), consisting of 7 sera each from 20 herds, submitted to the Michigan Department of Agriculture for regulatory purposes were kindly provided by Dr. Richard Gatzmeyer.

The immune reference serum was a pool of sera obtained from blood collected at 2 to 8 WPI from 3 experimentally infected animals. The nonimmune reference serum was obtained from blood of a specific-pathogen-free (SPF) pig, known to be *E. suis* free.

Indirect hemagglutination assay (IHA). The IHA procedure of Smith and Rahn (1975) was followed.

1) Preparation of sensitized sheep erythrocytes (S-RBC) with IHA antigen: Five milliliters of sheep blood was collected with equal volume of Alsever's solution and erythrocytes were washed 3 times with phosphate buffered saline (PBS; pH 7.2). A half milliliter of packed erythrocytes were suspended in 9.5 ml of PBS in a 50-ml

beaker and stirred on a magnetic stirrer. While stirring, 24.7 mg of IHA antigen (provided by Dr. A. R. Smith, University of Illinois, Champaign-Urbana, IL) was added followed by dropwise addition of 1 ml of 2.5% glutaraldehyde. The mixture was stirred for one hour, then 1 ml of 2% ovalbumin was added and the mixture was stirred for another 30 minutes. S-RBC were washed 3 times and resuspended in 9 times its volume with PBS and stored in liquid nitrogen. For use in the IHA, 10% S-RBC solution was diluted 20 times with PBS. The glutaraldehyde treated sheep erythrocytes (G-RBC) were prepared identical to S-RBC except no antigen was added. G-RBC was used as a 10% solution.

2) Assay: Test sera were heat inactivated at 56 °C for 30 minutes. To 100 µl of inactivated serum, 0.85 ml of PBS and 50 µl of the 10% G-RBC solution were added and the mixture was incubated for 15 minutes at 25 °C. The mixture was centrifuged at 500 g for 10 minutes and 50 µl of the supernatant was transferred to a round bottom 96 well microplate. The transferred sera were diluted serially (two fold) using 1% heat inactivated rabbit serum in PBS with a starting dilution of either 1:10 or 1:20. The test antigen, 50 µl of 0.5% S-RBC solution, was added to each well. The plate was covered and incubated at 4 °C over night.

The IHA titer (geometric mean for duplicate or triplicate samples) was interpreted as follows: 1:80 or more was positive; 1:20 or less was negative; and a titer between 1:20 to 1:80 was suspicious.

100K antigen preparation. Blood collected with citrate phosphate dextrose solution (14 ml/100 ml blood) containing 0.2% inosine was centrifuged at 500g for 20 minutes and the plasma and buffy coat were removed. Erythrocytes were washed 3 times with Eagle's minimum essential medium containing 0.2% inosine (EMI). After the final wash, erythrocytes were resuspended in the same volume of EMI containing 5% fetal calf serum. The suspension was mixed with 6.6% and 10% (v/v) of 40% PVP and glycerol, respectively, and stored at -80 °C. The frozen erythrocytes were thawed and centrifuged at 100,000g for 40 minutes. The pellet was resuspended in PBS (pH 7.4) and sequentially centrifuged at 500g for 20 minutes and at 10,000g and 100,000g for 30 minutes each. The pellet from the 100,000g centrifugation was solubilized by 3 times freeze-thawing in Tris buffer (pH 8.0) containing 5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1% Nonidet P-40 and 0.1% sodium dodecyl sulfate (SDS), and stored at -80 °C (100K antigen).

100K antigens were prepared from noninfected and infected blood (>75% parasitemia) collected before and after infection from the same animal (Es^- and Es^+ 100K antigens, respectively). The protein concentrations of the antigen preparations were measured by the Bio-Rad protein assay (Bio-Rad, Richmond, CA).

Enzyme-linked immunosorbent assay (ELISA). Two ELISA's were performed using either horse radish peroxidase (HRP) conjugated protein A (ProA-ELISA) or HRP conjugated rabbit anti-pig IgG (IgG-ELISA). The wells of polystyrene ELISA plates (Immulon 2, Dynatech Laboratories Inc., Chantilly, VA) were coated with 0.5 µg (ProA-ELISA) or 1 µg (IgG-ELISA) of the Es^+ or Es^- 100K antigens in 100 µl of 50 mM carbonate buffer (pH 8.5), sealed, and incubated at 37 °C over night. After coating the wells, the plates were washed 3 times with ELISA washing buffer, composed of PBS (pH 7.4) containing 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate), and blocked for one hour with 200 µl of 2% bovine serum albumin (BSA) in 50 mM carbonate buffer (pH 8.0). The plate was then incubated with 100 µl of the 1:100 dilution of the test sera, diluted with ELISA incubation buffer (0.5% BSA-PBS-0.05% Tween, pH 7.2), for 2 hours at 25 °C. The plate was washed 4 times with ELISA washing buffer, followed by the addition of 100 µl of 1:10,000 dilution of HRP conjugated protein A (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) diluted with ELISA incubation buffer, or 1:5,000 dilution of HRP conjugated rabbit anti-pig IgG (Sigma Chemical Co.) diluted with ELISA incubation buffer. The plates were then incubated for another hour at 25 °C. Following incubation, the plates were washed 4 times with the ELISA washing buffer. For the ProA-ELISA, plates were incubated with 100 µl of 0.03% ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid); Kirkegaard & Perry) in 50 mM phosphate citrate buffer

containing 0.01% hydrogen peroxide for 15 minutes. For the IgG-ELISA, plates were incubated with 100 µl of 0.01% ABTS (Sigma Chemical Co.) in 50 mM phosphate citrate buffer containing 0.01% hydrogen peroxide for 30 minutes. After incubation, the optical density (OD) of each well was measured at 405 nm by an ELISA reader (EIA Reader Model EL 307, Bio-Tek Instruments Inc., Burlington, VT).

The results were reported as two values:

1. CrudeEs-Test = the OD obtained with the Es^+ antigen;
2. NetEs-Test = the difference between the OD's obtained with the Es^+ antigen compared to the Es^- antigen, calculated by subtracting OD value of the Es^- antigen from the Es^+ antigen OD value.

Individual cut off values (mean + 2 standard deviations) were determined for each of the two ELISA's (CrudeEs-Test or NetEs-Test) using the data from the pre-infection sera obtained from the 8 experimentally infected pigs. The means and standard deviations of OD values of pre-infection sera and cut off values for each test are shown in Table 4-1. The positive/negative results of each CrudeEs-Test and NetEs-Test of the two ELISA's were determined as follows: in each test, sera showing OD's equal to or greater than the respective cut off value were classified as positive; and sera showing OD's less than the respective cut off value were classified as negative.

Table 4-1. Determination of cut off values used for classification of test sera to positive or negative by ProA (protein A)- and IgG-ELISA's and dot blot.

Assay	Test	Mean*	Standard deviation†	Cut off value‡
ProA-ELISA	CrudeEs-Test	0.202	0.053	0.308
	NetEs-Test	0.117	0.045	0.207
IgG-ELISA	CrudeEs-Test	0.079	0.023	0.125
	NetEs-Test	0.045	0.018	0.081
Dot blot 1§	CrudeEs-Test	1.460	0.518	2.496
	NetEs-Test	0.605	0.209	1.023
Dot blot 2‖	CrudeEs-Test	1.291	0.155	1.601
	NetEs-Test	0.281	0.268	0.817

* = Mean values of ELISA OD's or dot blot percent bindings of pre-infection sera obtained from blood of 8 experimentally infected pigs, determined by CrudeEs-Test or NetEs-Test.
† = Standard deviations of ELISA OD's or dot blot percent bindings of pre-infection sera determined by CrudeEs-Test or NetEs-Test.
‡ = determined by mean + 2 standard deviations.
§ = Using ^{125}I-protein A batch 1.
‖ = Using ^{125}I-protein A batch 2.

Dot blot (DB). For this assay, protein A was labeled with ^{125}I by the method of Fraker and Speck (1978). Three micrograms of 100K antigen (either Es^+ or Es^-) were placed in the respective wells of a blotter (Bio-Blot; Bio-Rad). Antigen was applied to the nitrocellulose membrane (NC) using vacuum and the wells were washed once with PBS (pH 7.4). The blotted NC was blocked overnight in blot blocking buffer, 2% BSA in tris buffered saline (TBS, pH 7.4) with 0.5% Tween 20. After blocking, the NC was cut into pairs of Es^+ and Es^- antigen dots. Each pair of dots were placed into the wells of 24 well culture plates (Corning, Corning, NY), and incubated with 0.5 ml of a 1:200 dilution of serum diluted with blot incubation buffer (0.5% BSA-TBS-0.5% Tween, pH 7.2) for 2 hours. The dots were then washed 3 times with blot washing buffer (0.2% BSA-TBS-0.5% Tween, pH 7.0), followed by incubation with 0.5 ml of ^{125}I-protein A (20 ng/ml in 0.5% BSA-TBS-0.5% Tween) for one hour. The dots were washed 3 times with blot washing buffer, the pairs were separated, and the radioactivity of each dot was measured in a gamma counter (Gamma Counting System, RIA 300; Tm Analytic, Elk Grove Village, IL). To adjust for the effect of radioactive decay over time, the percent binding was calculated by the following equation:

$$\text{Percent binding} = \frac{\text{Count per minute (CPM) of a dot}}{\text{CPM of total amount added to a well}} \times 100$$

As used in the ELISA, test results were determined by two methods:

1. CrudeEs-Test = the percent binding obtained with the Es^+ antigen dot;
2. NetEs-Test = the difference between the Es^+ and Es^- antigen dots, calculated by subtracting the percent binding with the Es^- antigen dot from the percent binding with the Es^+ antigen dot.

Individual cut off values (mean + 2 standard deviations) were determined for each CrudeEs-Test or NetEs-Test using pre-infection sera from 8 experimentally infected pigs. The means and standard deviations for percent binding of pre-infection sera and cut off values for each test are shown in Table 4-1. For performing DB, two batches of protein A were ^{125}I-iodinated separately. Because the ^{125}I-iodination efficiency of the two batches of protein A were different, cut off values for each batch were determined individually using the same sera (shown as dot blot 1 and 2 in Table 4-1). The cut off value of dot blot 1 was used for experiments evaluating sera from experimentally infected pigs. The cut off value of dot blot 2 was used for experiments evaluating field serum samples. The positive/negative results of each CrudeEs-Test and NetEs-Test were determined as follows: in each test, sera showing percent binding equal to or greater than the respective cut off values were classified as positive; and sera showing percent binding less than the respective cut off values were classified as negative.

Comparison of specificities of the anti-pig IgG and protein A reagents by western blot. To evaluate whether there were differences in the specificities of the rabbit anti-pig IgG and protein A reagents, western blotting using the 100K antigen was performed in parallel using HRP conjugated anti-pig IgG and ^{125}I labeled protein A.

The Es^+ and Es^- 100K antigens were mixed with sample buffer (100 mM Tris (pH 6.8), 3.3% (w/v) SDS, 16.7% (v/v) glycerol, 4.2% (v/v) β-mercaptoethanol, and a few crystals of bromophenol blue) and placed in boiling water for 3 minutes. SDS-polyacrylamide gel electrophoresis (PAGE) was performed with these antigens using a 9% gel (Smith, 1984). The proteins were transferred to NC's electrophoretically by the method of Towbin et al. (1979). The proteins transferred to the NC's were blocked with blot blocking buffer at 4 °C over night. The NC's were incubated with either immune or nonimmune reference sera diluted 1:100 with blot incubation buffer at 25 °C for two hours, followed by washing 3 times with blot washing buffer, 10 minutes each wash.

For IgG western blotting, the NC's were incubated with 1:2,000 dilution of HRP conjugated rabbit anti-pig IgG (Sigma Chemical Co.) diluted with blot incubation buffer for another one hour and washed 3 times with blot washing buffer. The NC's were finally incubated for two hours with triethanolamine buffered saline (pH 7.5) containing 0.05% 4-chloro-1-naphthol (Sigma Chemical Co.) and 0.01% hydrogen peroxide.

For protein A western blotting, the NC's were incubated with ^{125}I-protein A (10^5 CPM/ml in blot incubation buffer) at 25 °C for one hour. Following incubation, the NC's were washed 3 times with blot washing buffer and air dried. Direct autoradiography was performed with the dried NC's using Kodak X-omat RP film, over night exposure at -80 °C.

Protein standards used for molecular weight (MW) markers (SDS-6H; Sigma Chemical Co.) consisted of bovine erythrocyte carbonic anhydrase (MW 29,000), ovalbumin (MW 45,000), bovine plasma albumin (MW 66,000), rabbit muscle phosphorylase B (MW 97,400), *Escherichia coli* β-galactosidase (MW 116,000) and rabbit muscle myosin (MW 205,000). The transferred MW standards were visualized by India ink stain (Hancock & Tsang, 1983).

Western blot analysis of field samples. Of the 140 field sera tested by the two ELISA's, DB and IHA, 63 samples were selected for western blot analysis so that representative numbers of sera yielding different combinations of results in the three new assays (ProA- and IgG-ELISA's and DB) were included. The selection procedure was as follows: The 140 sera were stratified into 6 groups by combinations of the positive/negative results of ProA-ELISA CrudeEs-Test, IgG-ELISA CrudeEs-Test and DB CrudeEs-Test (all ProA-ELISA positive sera were also positive in IgG-ELISA). The cut off values used to stratify the sera were the mean OD or percent binding of pre-infection sera from the 8 experimentally

infected pigs plus 3 standard deviations, instead of 2 standard deviations, to increase test specificity so that only highly positive samples in each assay could be classified as positive in the selection process. A similar number of sera were selected from each of the 6 combinations.

The procedures of SDS-PAGE and western blotting were done as described above with the following modifications. SDS-PAGE was performed using a 9% gel consisting of two wide lanes, one with Es^+ and the other with Es^- 100K antigen, and a lane for MW standards. After electrophoresis and electrotransfer of the proteins to NC, the NC was blocked with blot blocking buffer at 4 °C over night. The NC was then cut into narrow strips of Es^+ and Es^- 100K antigens. The strips were incubated individually with the serum sample, diluted 1:100 with blot incubation buffer, at 25 °C for two hours (one Es^+ and one Es^- antigen strip for each sample), and washed 3 times with blot washing buffer, 10 minutes each wash. The NC strips were incubated with HRP conjugated rabbit anti-pig IgG for one hour, then washed 3 times with blot washing buffer, and were then incubated for two hours with triethanolamine buffered saline (pH 7.5) containing 0.05% 4-chloro-1-naphthol and 0.01% hydrogen peroxide. The blots were evaluated for the presence of *E. suis* specific antigen bands at MW's 24, 37, 47, 71, 81, 101 and 106 kd, as presented in Chapter 3.

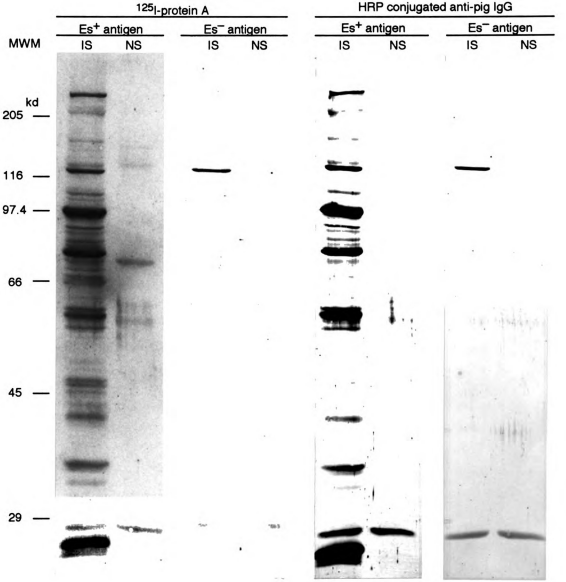
Statistical analysis. Statistical analysis was done using the computer program StatView (version 4.0, Abacus Concepts, Berkeley, CA). Chi-square contingency analysis was performed to evaluate the associations between each positive/negative result of the two ELISA's, the DB and the IHA, and the associations of the two ELISA's, the DB and the IHA positive/negative results with the detection of each *E. suis* specific antigens in the IgG western blots. For 2x2 contingency analysis with expected values less than 5, a continuity correction was made (Gill, 1978). A P value less than 0.05 was considered significant.

Results

Comparison of western blots using anti-pig IgG and Protein A. The results of western blots using Es^+ and Es^- 100K antigens, immune and nonimmune reference sera, and HRP conjugated rabbit anti-pig IgG and ^{125}I labeled protein A are summarized in Figure 4-1. No differences were detected in the bands present in the western blots developed with either rabbit anti-pig IgG or Protein A.

Serum samples from experimentally infected pigs. Triplicate samples of pre- and post-infection sera obtained from 8 experimentally infected pigs were tested by ProA- and IgG-ELISA, DB and IHA. The positive/negative results of each assay are summarized in Table 4-2. The two ELISA's and DB exhibited a high degree of sensitivity for detecting antibody responses in post-infection sera whether evaluated by CrudeEs-Test (94% to 96%) or NetEs-Test (91% to 96%). The pre-infection sera were negative in all tests, except for one sample that was positive in the IgG-ELISA NetEs-Test. Negative results with post-infection sera in the two ELISA's and DB tended to occur with sera collected during the early stage of infection. However, more than half of the 1 WPI sera were positive in the ProA- and IgG-ELISA's and DB (71, 63 and 88% sera were positive by CrudeEs-Test, and 57, 75 and 88% sera were positive by NetEs-Test, respectively). Eighty-

Figure 4-1. Comparison of antibody detection by protein A and rabbit anti-pig IgG by western blot. Western blotting were performed using *Eperythrozoon suis* positive (Es^+) and negative (Es^-) 100K antigens and immune (IS) and nonimmune (NS) reference sera. Antibodies bound to antigens were detected by ^{125}I-protein A (10^5 CPM/ml of the buffer) or horse radish peroxidase (HRP) conjugated rabbit anti-pig IgG (1:2000 diluted in the buffer). ^{125}I-protein A treated nitrocellulose membranes (NC's) were exposed to Kodak X-omat RP film over night at -80 °C. HRP conjugated anti-pig IgG treated NC's were incubated with hydrogen peroxide containing 4-chloro-1-naphthol for two hours. MWM = Molecular weight marker.


125I-protein A
HRP conjugated anti-pig IgG
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Es− antigen
Es+ antigen
Es− antigen
MWM
IS
NS
IS
NS
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IS
NS
kd
205
116
97.4
66
45
29

Figure 4-1.

Table 4-2. The results of ProA (protein A)- and IgG-ELISA's, dot blot and indirect hemagglutination assay (IHA) with sera obtained from 8 experimentally infected pigs at pre-infection and various post-infection time*.

	ProA-ELISA		IgG-ELISA		Dot blot		IHA
Serum	CrudeEs-Test	NetEs-Test	CrudeEs-Test	NetEs-Test	CrudeEs-Test	NetEs-Test	
Before infection	0/8 †	0/8	0/10	1/10	0/10	0/10	0/10
1 WPI‡	5/7	4/7	5/8	6/8	7/8	7/8	0/8
2	6/7	6/7	7/7	7/7	6/7	6/7	6/7
3	8/8	8/8	8/8	8/8	8/8	8/8	8/8
4	8/8	7/8	8/8	7/8	8/8	8/8	8/8
6	8/8	8/8	8/8	8/8	8/8	8/8	8/8
8	5/5	5/5	5/5	5/5	5/5	5/5	5/5
10	6/6	6/6	6/6	6/6	6/6	6/6	6/6
12	4/4	4/4	4/4	4/4	4/4	4/4	4/4
Total§	50/53	48/53	51/54	51/54	52/54	52/54	45/54

* = In IgG-ELISA, dot blot and IHA, 2 additional pre-infection sera collected from 2 pigs at different times were evaluated. The total number of samples varied among post-infection period, because samples were missed due to pig withdrawals from the study.
† = The number of positive samples/the number of samples.
‡ = Number of weeks post-infection.
§ = The total of post-infection sera only.

three percent of the post-infection sera were positive by IHA, although none of the 1 WPI sera were positive by IHA.

Changes in OD values measured by the two ELISA's, percent binding in the DB, and the log IHA titer during the course of infection are presented in Figure 4-2. In the two ELISA's and DB, the assay values (the OD or percent binding) were expressed in 3 ways: CrudeEs-Test, NetEs-Test and assay value obtained with Es^- antigen only (N-Ag value). CrudeEs-Test OD values in either ELISA demonstrated a rapid increase during the first 4 WPI, followed by a decrease during 4 to 6 WPI, and then increased again after 6 WPI. On the other hand, NetEs-Test OD values in either ProA- or IgG-ELISA demonstrated a rapid increase during the first 2 to 3 WPI and then a gradual increase through the 12 WPI. Consequently, the differences in OD values between CrudeEs-Test and NetEs-Test in either ELISA were the largest at 4 WPI, coincident with a peak in N-Ag values at 4 WPI in either ELISA. Both DB CrudeEs-Test and NetEs-Test demonstrated a rapid increase in percent binding during the first two weeks after infection and a gradual decrease after 3 WPI. The DB N-Ag value gradually increased during the first 4 WPI and then gradually decreased after 4 WPI. With the IHA, a small increase in the log IHA titer was found in the first WPI, followed by a rapid increase during 2 to 4 WPI, and then a gradual decrease thereafter except for a second small peak at 10 WPI. The time course of IHA titer development during the experimental period was similar to N-Ag values in the two ELISA's; a small

Figure 4-2. The ProA (protein A)- and IgG-ELISA's, dot blot and indirect hemagglutination assay (IHA) with sera of experimetally infected pigs. Pre-infection and 1, 2, 3, 4, 6, 8, 10 and 12 weeks post-infection (WPI) sera from 8 experimentally infected pigs were assayed with the ProA-ELISA (A), IgG-ELISA (B), dot blot (C) and IHA (D). In the two ELISA's and dot blot, the mean optical densities (OD) and percent binding in CrudeEs-Test and NetEs-Test were presented in addition to the mean OD's and percent binding of the ELISA's and dot blot using Es^- antigen (N-Ag value). IHA titer was converted to $\log_2$ (x/10). The dash lines show the cut off values of CrudeEs-Test and NetEs-Test. Error bars represent standard deviations of each value.

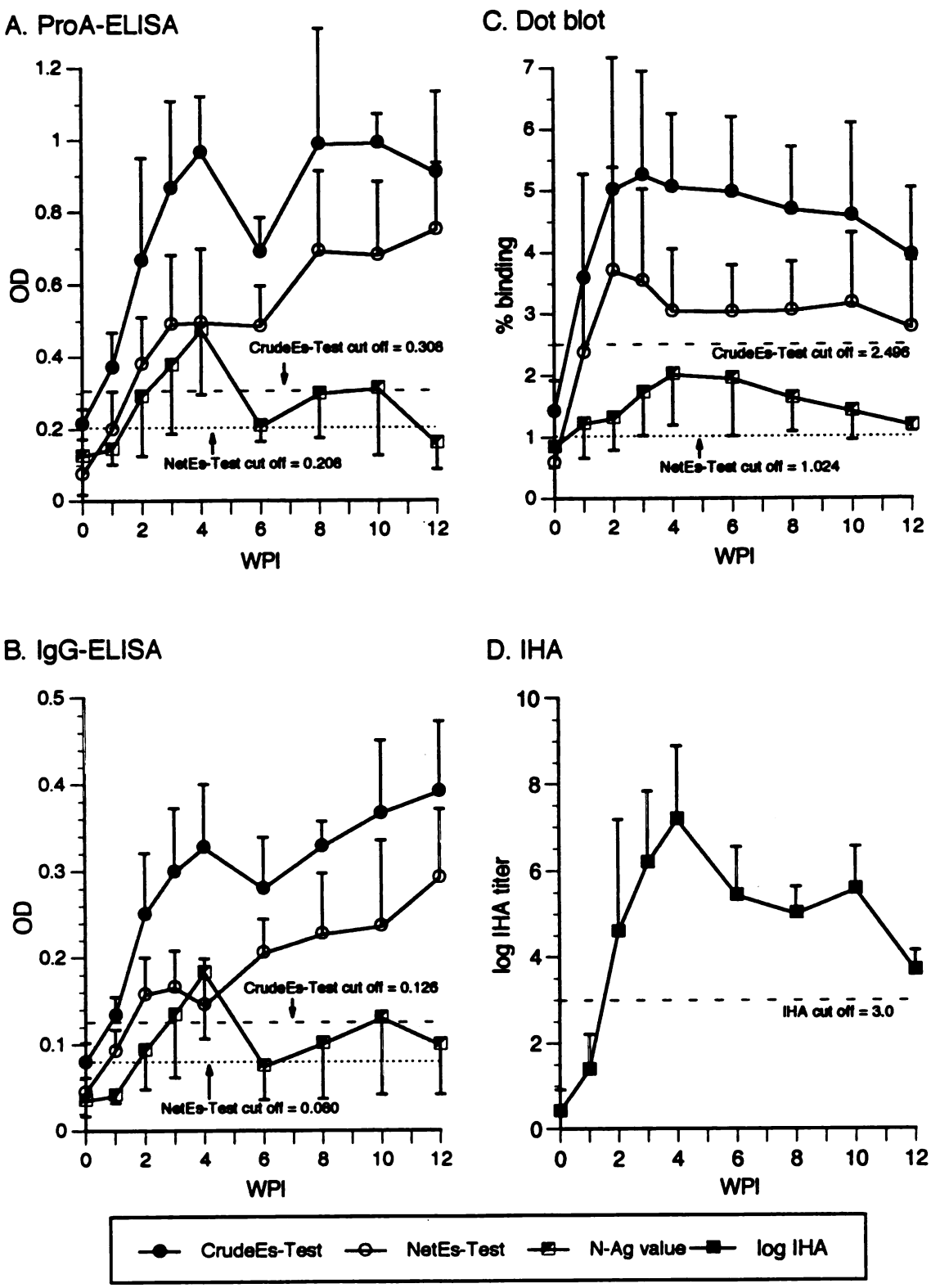
A. ProA-ELISA
OD
WPI
CrudeEs-Test cut off = 0.308
NetEs-Test cut off = 0.208
C. Dot blot
% binding
WPI
CrudeEs-Test cut off = 2.496
NetEs-Test cut off = 1.024
B. IgG-ELISA
OD
WPI
CrudeEs-Test cut off = 0.126
NetEs-Test cut off = 0.060
D. IHA
log IHA titer
WPI
IHA cut off = 3.0
CrudeEs-Test
NetEs-Test
N-Ag value
log IHA

Figure 4-2.

increase in OD values during the first WPI, followed by a rapid increase during 2 to 4 WPI, then a gradual decrease with a small second peak at 10 WPI.

Field serum samples. A total of 140 field sera were tested in duplicate by ProA- and IgG-ELISA's, DB and IHA. The positive/negative results of these assays are summarized in Table 4-3. In each ProA- or IgG-ELISA, or DB, there was a large difference in the numbers of positive sera detected by CrudeEs-Test versus NetEs-Test. The difference in the numbers of positive sera between CrudeEs-Test and NetEs-Test were 46 (33% of the total sera tested) in the ProA-ELISA, 49 (35%) in the IgG-ELISA and 73 (52%) in the DB. Considering all 3 assays, 14 of the 140 samples were positive by all CrudeEs-Test's but negative by all NetEs-Test's in ProA- and IgG-ELISA, and DB. When comparisons were made between the two ELISA's and DB, IgG-ELISA exhibited considerably greater numbers of positive serum samples than ProA-ELISA and DB, regardless of being evaluated by CrudeEs-Test or NetEs-Test. In IHA, only 14 sera were *E. suis* positive and an additional 46 sera were classified *E. suis* suspicious. Only 13 sera were negative in all ProA- and IgG-ELISA's, DB and IHA.

Table 4-4 lists the association level between the positive/negative results of each ELISA, the DB and the IHA. In each CrudeEs-Test and NetEs-Test, the positive/negative results of the two ELISA's and DB were significantly ($P < 0.001$) associated with each other as determined by Chi-square

Table 4-3. The number of positive field sera detected by CrudeEs-Test or NetEs-Test in ProA (protein A)- and IgG-ELISA's and dot blot, and by indirect hemagglutination assay (IHA).

	ProA-ELISA		IgG-ELISA		Dot blot		IHA*	
Total tested	CrudeEs-Test	NetEs-Test	CrudeEs-Test	NetEs-Test	CrudeEs-Test	NetEs-Test	Pos	Sus
140 serum	70 (50%)	24 (17%)	127 (91%)	78 (56%)	86 (61%)	13 (9%)	14 (10%)	46 (33%)

* = IHA results were expressed as the number of positive or suspicious sera.

Table 4-4. P values of Chi-square contingency analysis between the positive/negative results of ProA (protein A)-ELISA, IgG-ELISA, dot blot and indirect hemagglutination assay (IHA).

Comparison	CrudeEs-Test	NetEs-Test
ProA-ELISA vs IgG-ELISA	.0002	<.0001
ProA-ELISA vs dot blot	<.0001	<.0001
IgG-ELISA vs dot blot	<.0001	.0007
IHA vs ProA-ELISA	.2516	.0680
IHA vs IgG-ELISA	.0073	.3264
IHA vs dot blot	.1066	.9567

contingency analysis. However, the results of IHA were not significantly associated with the results of the other assays (two ELISA's or DB), except with CrudeEs-Test of IgG-ELISA in which all of the IHA positive and suspicious sera were positive in CrudeEs-Test.

Western blot analysis of the field samples. The number of sera selected for western blot analysis from each combination of positive/negative results determined by ProA- and IgG-ELISA and DB CrudeEs-Test's are shown in Table 4-5. The 63 selected field samples were evaluated for their specificity to 7 *E. suis* specific antigens using IgG western blotting. The relationships of the positive/negative results of the two ELISA's, DB or IHA with the total number of the *E. suis* specific antigen bands detected in the western blots are summarized in Table 4-6. All the sera reacted with at least one of the 7 *E. suis* specific antigen bands. However, only

Table 4-5. Field sera in combinations of positive/negative results determined by ProA (protein A)-ELISA, IgG-ELISA and dot blot CrudeEs-Test*.

ProA-ELISA	IgG-ELISA	Dot blot	Number of field sera	Number of selected sera†
Positive	Positive	Positive	12	11
Positive	Positive	Negative	11	9
Negative	Positive	Positive	24	10
Negative	Positive	Negative	64	14
Negative	Negative	Positive	2	2
Negative	Negative	Negative	27	17
Total			140	63

* = Cut off values used were mean optical density or percent binding of pre-infection sera obtained from blood of 8 experimentally infected pigs plus 3 standard deviations.
† = Number of sera selected for western blot analysis.

one serum sample reacted with all of 7 *E. suis* specific antigen bands.

In the ProA-ELISA, all positive sera, either by CrudeEs-Test or NetEs-Test, recognized 3 or more *E. suis* specific antigen bands. Comparing the positive sera as determined by CrudeEs-Test versus NetEs-Test, considerable differences, as consequently shown by the positive-negative result combination in CrudeEs-Test-NetEs-Test, were observed in the number of sera that recognized 3 to 5 of the 7 *E. suis* antigen bands. Fewer sera that recognized 3 to 5 *E. suis* antigen bands were positive in NetEs-Test compared to that in CrudeEs-Test (6 vs. 27, respectively). Sera that recognized 6 or all 7 antigens were positive in both CrudeEs-Test and

Table 4-6. The number of positive and negative field sera as determined by ProA (protein A)- and IgG-ELISA's, dot blot and indirect hemagglutination assay (IHA), stratified by the total number of 7 *E. suis* specific antigen bands detected in IgG western blots.

			Total number of bands detected in the western blots						
Assay	result	Total	1	2	3	4	5	6	7
ProA-ELISA									
CrudeEs-Test	Positive	38	0	0	10	8	9	10	1
	Negative	25	2	10	7	5	1	0	0
NetEs-Test	Positive	16	0	0	2	2	2	9	1
	Negative	47	2	10	15	11	8	1	0
CrudeEs-Test-	Pos-Pos	16	0	0	2	2	2	9	1
NetEs-Test	Pos-Neg	22	0	0	8	6	7	1	0
combination*	Neg-Neg	25	2	10	7	5	1	0	0
IgG-ELISA									
CrudeEs-Test	Positive	56	2	4	16	13	10	10	1
	Negative	7	0	6	1	0	0	0	0
NetEs-Test	Positive	32	0	0	6	8	7	10	1
	Negative	31	2	10	11	5	3	0	0
CrudeEs-Test-	Pos-Pos	32	0	0	6	8	7	10	1
NetEs-Test	Pos-Neg	24	2	4	10	5	3	0	0
combination*	Neg-Neg	7	0	6	1	0	0	0	0
Dot blot									
CrudeEs-Test	Positive	40	0	1	12	7	9	10	1
	Negative	23	2	9	5	6	1	0	0
NetEs-Test	Positive	10	0	0	1	1	2	6	0
	Negative	53	2	10	16	12	8	4	1
CrudeEs-Test-	Pos-Pos	10	0	0	1	1	2	6	0
NetEs-Test	Pos-Neg	30	0	1	11	6	7	4	1
combination*	Neg-Neg	23	2	9	5	6	1	0	0
IHA	Positive	5	0	1	1	0	2	1	0
	Suspicious	21	0	2	5	3	5	6	0
	Negative	37	2	7	11	10	3	3	1

* = Combination of the results (Pos=positive and Neg=negative) of CrudeEs-Test and NetEs-Test.

NetEs-Test, except for one NetEs-Test positive sample that recognized 6 bands. Therefore with ProA-ELISA, NetEs-Test exhibited similar sensitivity, but higher specificity, compared to the CrudeEs-Test for detecting sera that reacted with 6 or more *E. suis* antigen bands.

In the IgG-ELISA, positive samples by CrudeEs-Test recognized a variety of *E. suis* specific antigen bands, ranging from 1 to all 7 bands. With positive sera as determined by CrudeEs-Test and NetEs-Test being compared, differences, as consequently shown by the positive-negative result combination in CrudeEs-Test-NetEs-Test, were observed in the number of sera that recognized 1 to 5 *E. suis* antigens. Fewer sera that recognized 1 to 5 bands were positive in NetEs-Test compared to CrudeEs-Test (21 vs. 45, respectively). Sera that recognized 6 or all 7 bands were positive in both CrudeEs-Test and NetEs-Test. IgG-ELISA NetEs-Test also showed similar sensitivity to but higher specificity than IgG-ELISA CrudeEs-Test for detecting sera that reacted 6 or more bands.

In DB, most of the positive samples in CrudeEs-Test and NetEs-Test recognized 3 or more *E. suis* specific antigen bands. Comparing positive sera as determined by CrudeEs-Test and NetEs-Test, considerable differences, as consequently shown by the positive-negative result combination in CrudeEs-Test-NetEs-Test, were observed in the number of sera that recognized 3 to 5 of 7 bands. Fewer sera that recognized 3 to 5 *E. suis* antigens were positive in NetEs-Test compared to

CrudeEs-Test (4 vs. 28, respectively). The number of positive sera that recognized 6 or more bands was 11 with CrudeEs-Test, but only 6 with NetEs-Test. The DB NetEs-Test also showed the same tendencies observed with the two ELISA's; similar sensitivity but higher specificity for detecting sera that reacted with 6 or more *E. suis* antigen bands than CrudeEs-Test.

In IHA, no clear relationships between the positive/negative results and the total number of *E. suis* specific antigen bands detected in the western blots was apparent.

Recognition of each *E. suis* specific antigen band by the field serum samples in western blots are summarized in Table 4-7 and 4-8. Of the 63 sera tested, the 24 and 106 kd antigens were recognized by 62 (98%) and 61 (97%) sera, respectively (Table 4-7). Conversely, the 81 kd antigen was recognized by only 11 (17%) sera. Considering the associations of test results and band recognitions in western blots, each *E. suis* antigen bands were recognized by a varied number of positive and negative sera as determined by CrudeEs-Test or NetEs-Test in each ELISA, and DB, and by IHA (Table 4-7). Statistical analysis of the associations between detecting each *E. suis* specific antigen in western blots, and the positive/negative results of each ELISA, DB and IHA are described in Table 4-8. Detection of either 37, 47, 71, 81 or 101 kd antigens were significantly ($P < 0.05$) associated with the positive/negative results of NetEs-Test

in the two ELISA's although the levels of associations (contingency coefficients) were relatively low. In addition, significant associations between the detection of *E. suis* specific antigen bands in western blots with the positive/negative results were observed in the 37 kd antigen with CrudeEs-Test's in ProA- and IgG-ELISA and DB, 47 kd antigen with ProA-ELISA CrudeEs-Test, and DB CrudeEs-Test and NetEs-Test, and 101 kd antigen with ProA-ELISA CrudeEs-Test and DB CrudeEs-Test. However, the results of IHA did not show any significant associations with the detection of any *E. suis* specific antigens.

Table 4-7. Positive or negative field sera as determined by ProA (protein A)- and IgG-ELISA's, dot blot and indirect hemagglutination assay (IHA), that reacted to each *E. suis* specific antigen band.

				Molecular weight (kd) of the *E. suis* specific antigens						
Assay	Test	Result	Total	24	37	47	71	81	101	106
ProA-ELISA	CrudeEs-Test	Positive	38	38(100%)*	38(100%)	23 (61%)	12 (32%)	9 (24%)	17 (45%)	38(100%)
		Negative	25	24 (96%)	11 (44%)	1 (4%)	3 (12%)	2 (8%)	4 (16%)	23 (92%)
	NetEs-Test	Positive	16	16(100%)	16(100%)	14 (88%)	8 (50%)	6 (38%)	10 (63%)	16(100%)
		Negative	47	46 (98%)	33 (70%)	10 (21%)	7 (15%)	5 (11%)	11 (23%)	45 (96%)
IgG-ELISA	CrudeEs-Test	Positive	56	55 (98%)	48 (86%)	24 (43%)	15 (27%)	11 (20%)	21 (38%)	54 (96%)
		Negative	7	7 (100%)	1 (14%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	7 (100%)
	NetEs-Test	Positive	32	31 (97%)	31 (97%)	19 (59%)	13 (41%)	10 (31%)	17 (53%)	32(100%)
		Negative	31	31(100%)	18 (58%)	5 (16%)	2 (6%)	1 (3%)	4 (13%)	29 (94%)
Dot blot	CrudeEs-Test	Positive	40	40(100%)	38 (95%)	22 (55%)	11 (28%)	10 (25%)	18 (45%)	40(100%)
		Negative	23	22 (96%)	11 (48%)	2 (9%)	4 (17%)	1 (4%)	3 (13%)	21 (91%)
	NetEs-Test	Positive	10	10(100%)	10(100%)	8 (80%)	5 (50%)	4 (40%)	6 (60%)	10(100%)
		Negative	53	52 (98%)	39 (74%)	16 (30%)	10 (19%)	7 (13%)	15 (28%)	51 (96%)
IHA		Positive	5	5 (100%)	4 (80%)	3 (60%)	1 (20%)	1 (20%)	2 (40%)	5 (100%)
		Suspicious	21	21(100%)	19 (90%)	11 (52%)	6 (29%)	6 (29%)	9 (43%)	21(100%)
		Negative	37	36 (97%)	26 (70%)	10 (27%)	8 (22%)	4 (11%)	10 (27%)	35 (95%)
Total sera			63	62 (98%)	49 (78%)	24 (38%)	15 (24%)	11 (17%)	21 (33%)	61 (97%)

* = percentage of samples in a total of positive or negative sera determined by each test.

Table 4-8. Chi-square contingency analysis between the positive/negative results of field samples as determined by ProA (protein A)- and IgG-ELISA's, dot blot and indirect hemagglutination assay (IHA), and the detection of each *E. suis* specific antigen band in IgG western blots.

			Molecular weight (kd) of the *E. suis* specific antigen bands						
Assay	Test	Analysis	24	37	47	71	81	101	106
ProA-ELISA	CrudeEs-Test	P value	.8317	<.0001*	<.0001*	.0743	.2058	.0179*	.2995
		Contingency Coef.†	(.155)	(.550)	(.495)	(.219)	(.198)	(.286)	(.218)
	NetEs-Test	P value	>.9999	.0334*	<.0001*	.0121*	.0391*	.0042*	.9895
		Contingency Coef.	(.074)	(.298)	(.510)	(.338)	(.294)	(.340)	(.105)
IgG-ELISA	CrudeEs-Test	P value	>.9999	.0001*	.0737	.2722	.4457	.1190	>.9999
		Contingency Coef.	(.045)	(.475)	(.267)	(.194)	(.161)	(.243)	(.064)
	NetEs-Test	P value	>.9999	.0002*	.0004*	.0015*	.0034*	.0007*	.4584
		Contingency Coef.	(.124)	(.423)	(.407)	(.372)	(.346)	(.392)	(.181)
Dot blot	CrudeEs-Test	P value	.7776	<.0001*	.0003*	.3644	.0829	.0096*	.2505
		Contingency Coef.	(.165)	(.479)	(.417)	(.114)	(.253)	(.310)	(.232)
	NetEs-Test	P value	>.9999	.1532	.0088*	.0863	.1112	.1130	>.9999
		Contingency Coef.	(.055)	(.226)	(.351)	(.258)	(.250)	(.239)	(.078)
IHA		P value	.6998	.2039	.0927	.8187	.2280	.4450	.4840
		Contingency Coef.	(.106)	(.219)	(.265)	(.079)	(.212)	(.158)	(.150)

* = Significant associations ($P < 0.05$).
† = Contingency coefficient

Discussion

Reagents

In the developed assays, anti-pig IgG or protein A were used to detect immunoglobulins bound to antigens. Protein A has been shown to bind to IgG of most mammalian species and also to IgM, IgA and IgE with lower reactivity (Lindmark et al., 1983). It has been postulated that protein A binding to IgG occurs via the Fc portion of IgG and binding to other classes of immunoglobulins is mainly via the Fab portion. In pigs, protein A binds effectively to the main IgG subclasses, IgG_1 and IgG_2 (more than 90% reactivity to both subclasses), and less effectively to IgM (35 to 40% reactivity) and IgA (23% reactivity) (Lindmark et al., 1983). The anti-pig IgG used in the study was affinity purified from the sera of rabbits that was immunized with the whole molecule of pig IgG. Since the light chain (kappa or lambda chain) is shared by all classes of immunoglobulins, antibodies against the whole molecule of IgG could potentially react to all classes of immunoglobulins. Therefore, both reagents, protein A and anti-pig IgG, could recognize IgM to some degree but the degree of specificities of the two reagents for detecting IgM are probably different.

In the first experiment, the band appearances in the western blots using HRP conjugated anti-pig IgG and ^{125}I-protein A were compared to evaluate the specificity and the

suitability of two reagents for use in the immunoassays. No differences were found in the specificity of anti-pig IgG and protein A ability to detect antigen bands in western blots, for the band pattern and band intensity in the western blots using either anti-pig IgG or protein A were identical.

Experimental Infection

In the experimental infection, the proposed ELISA's and DB detected an antibody response sooner in post-infection sera from experimentally infected animals than the IHA. Two test methods, CrudeEs-Test and NetEs-Test, were evaluated in each ELISA and DB. The two tests showed similar sensitivities for detecting an antibody response in post-infection sera of experimentally infected pigs. CrudeEs-Test represented the IgG binding to the Es^+ antigen, while NetEs-Test represented the difference of IgG binding to Es^+ and Es^- antigens, thus more specifically representing IgG binding to *E. suis* specific antigens. Therefore, NetEs-Test should eliminate the effects of autoantibodies induced by *E. suis* infection and also erythrocyte antigens in the inoculum. It is assumed that the increase in OD or percent binding values in NetEs-Test of the two ELISA's or DB could represent a specific IgG immune response in the experimentally infected pigs with *E. suis*.

Indeed, the autoantibody production in pigs experimentally infected with *E. suis* was demonstrated in Figure 4-2. The increase in OD values and percent binding of the two ELISA's and DB using the Es^- antigen preparation after infection are potential evidence of an immune response to host derived proteins. Similar observations were reported in experimental infections with *Anaplasma* and *Babesia* when infected blood was used as an inoculum (Barbet et al., 1983; Budden & Dimopoullos, 1977; Callow & Dalgliesh, 1982; Goodger et al., 1985). It was suggested that isoerythrocyte antibodies were produced as a result of host immune reaction to isoerythrocyte antigens in the inoculum (Barry et al., 1986; Dimmock, 1973; Duzgun et al., 1988). In studies directed at the development of immunoassays for hemoparasitic infection using crude antigen preparations, it is expected that an apparent high sensitivity for detecting post infection sera may be interpreted by a reaction of isoerythrocytic antibodies to impurities of antigen preparation. Budden and Dimopoullos (1977) suggested that a capillary-tube agglutination test developed for anaplasmosis (Ristic, 1962) had detected only antibodies directed at erythrocytes, since purified *Anaplasma* bodies were not agglutinated by the infected serum, while erythrocyte stromata separated from *Anaplasma* bodies was agglutinated by the infected serum. In addition to isoerythrocyte antibodies directed at non-self erythrocytes, the development of autoantibodies to host self erythrocytes has been observed as

a form of cold agglutinin in hemoparasite infections such as eperythrozoonosis, haemobartonellosis, anaplasmosis, malaria and babesiosis, which are also associated with anemia (Bellamy et al., 1978; Oki & Miura, 1970; Schroeder & Ristic, 1965; Soni & Cox, 1974; Soni & Cox, 1975; Thoongsuwan & Cox, 1973; Zachary & Smith, 1985; Zulty & Kociba, 1990).

To understand the effect of antibodies directed at erythrocytes in diagnosis for bovine anaplasmosis, Duzgun et al. (1988) developed a similar ELISA system to the NetEs-Test in the present study, using 2 different antigens prepared before and after infection from the same animals (named positive and negative antigens, respectively). The "net reading", calculated by subtracting the negative antigen result from the positive antigen result, was evaluated for *A. marginale* infection. This test showed excellent specificity for it could differentiate between the sera of animals experimentally infected with *B. bovis* or *B. bigemina*. If the negative antigen test results were not subtracted from the positive antigen test results, 37% of the sera would have been falsely classified as positive for *A. marginale* infection. In swine eperythrozoonosis, a similar test system using positive and negative antigens in ELISA has been developed using lysates of the whole noninfected and infected erythrocytes as ELISA coating antigens (Schuller et al., 1990).

The N-Ag values plotted in Figure 4-2 illustrate autoantibody development during the course of infection. The

N-Ag values demonstrated an initial peak at 4 WPI in each ELISA and DB. Thus an observed rapid increase in the OD or percent binding value of CrudeEs-Test in either ELISA or DB during the first 4 WPI was largely reflected by the autoantibody development. This demonstrates the possible bias, caused by autoantibodies, in the interpretation of specific humoral immune response against hemoparasites if antibodies are measured by an assay using a crude antigen prepared only from infected blood (Es^+ antigen).

In the two ELISA's and DB, more than 90% of post-infection sera of experimentally infected animals were positive by both CrudeEs-Test and NetEs-Test. Most of the negative results were observed in the sera of early infection stage, presumably because of the time required for the host immune system to respond to *E. suis* infection. With the IHA, 1 WPI sera were all negative. It has been reported that IHA measures IgM titer. Generally, with humoral immune responses in a primary infection, IgM levels rise first followed by increasing IgG levels. The failure of IHA to detect 1 WPI sera may be due to its inability to detect low concentrations of IgM. However, considering the observation in Chapter 3 that the IHA antigen consisted of mainly host proteins and that antibodies reacting to the IHA antigen were mainly autoantibodies, the failure of IHA to detect 1 WPI sera could be due to a delay of autoantibody production after *E. suis* infection. This is consistent with the hypothesis that autoantibody production may be induced by alterations of

erythrocyte membrane proteins or the exposure of hidden erythrocyte antigens due to *E. suis* infection (Zachary & Smith, 1985). Evidence supporting the hypothesis that IHA may detect only autoantibodies is found in Figure 4-2; the observation that IHA titer development was identical to N-Ag value development in both ELISA's suggests that IHA and ELISA's using Es^- antigen detected the same antibody, presumably autoantibodies to host derived antigens.

Hsu et al. (1992) observed a high correlation (r=0.8601) between log IHA titers and ELISA OD values using an antigen prepared from an infected erythrocyte lysate. In their procedure, lysed infected erythrocytes were centrifuged at 1000g and the supernatant discarded. The pellet was suspended in PBS and frozen. The frozen suspension was thawed and centrifuged at 11000g. The resultant supernatant was used for the coating antigen in ELISA. Their results are inconsistent with the results obtained here, in which the log IHA titers and OD values of CrudeEs-Test and NetEs-Test in the two ELISA's and DB did not take similar courses during the experimental period. The correlation between the log IHA titers and the OD values of their ELISA may indicated that their ELISA was primarily detecting autoantibodies.

FIELD SERA

The results of testing sera from naturally exposed pigs showed considerable variation among the 4 assays. The numbers of positive samples in CrudeEs-Test and NetEs-Test in each ELISA's or DB were quite different. This indicates that a considerable amount of antibodies reactive to the Es⁻ antigen (autoantibodies or antibodies crossreactive to host antigens) had been produced in pigs raised under field condition, regardless of *E. suis* infection status. A possible explanation of this observation is that animals in the field are exposed to various etiologic agents which could induce development of agent specific antibodies, antibodies crossreactive to host antigens due to the similarity of agents and host antigens, and sometimes non-specific antibodies via polyclonal B cell activation. Induction of autoantibodies as a result of polyclonal B cell activation has been commonly observed in parasitic diseases including trypanosomiasis, leishmaniasis, malaria, babesiosis and schistosomiasis (Cohen, 1982). Therefore, it is likely that antibodies reactive or crossreactive to host antigens may develop in pigs under field conditions.

This hypothesis was supported by the result of the subsequent western blot experiments with the field serum samples. In several western blots, strong bands were observed at different molecular weight positions from *E. suis* specific antigens (data not shown). These antigens were

determined to be derived from the host because the bands were observed in both Es^+ and Es^- antigen preparations. Under field conditions, the presence of anti-erythrocyte antibodies (IgM and IgG) not associated with *E. suis* infection has been observed in sows with nonhemolytic anemia (van Leengoed et al., 1992). Disagreements between experimentally infected animals versus animals infected in field conditions have been reported previously with *A. marginale*. Serotests for bovine anaplasmosis such as complement fixation, capillary tube agglutination, card agglutination and indirect fluorescent antibody tests were more accurate for diagnosing experimental infections than for diagnosing field infections (Gonzalez et al., 1978; Rogers, 1971).

The IgG-ELISA of this study classified a greater number of field sera as positive compared to the ProA-ELISA and the DB, regardless of whether the results were evaluated by CrudeEs-Test or NetEs-Test (Table 4-3). The percentage of the positive sera in IgG-ELISA CrudeEs-Test and NetEs-Test were 91 and 56%. On the other hand, the percentage of the positive sera in ProA-ELISA CrudeEs-Test and NetEs-Test were 50 and 17%. Since true status of *E. suis* infection of field sera is difficult to assess, the ability of those tests to detect true infection status (sensitivity and specificity) could not be evaluated in this study and thus neither could the true percentage of the *E. suis* positive sera be determined (true prevalence). Unfortunately, definite diagnosis of *E. suis* infection is possible only by detection

of parasitemia in acutely affected, febrile animals (Smith, 1981). The duration of the acute parasitemic phase is short and the organism can not be detected in animals that have recovered from the febrile stage (Splitter, 1951). Even though there was a disagreement of the number of positive sera detected by the two ELISA's and DB, the three assays showed a similar ability to distinguish positive and negative sera, based on the contingency analysis of the positive/negative results (Table 4-4). In another words, positive sera determined by one of the two ELISA's or DB tended to be positive in other tests.

Western Blot Analysis

When the recognitions of each *E. suis* specific antigen by the field serum samples in the western blots were compared, all the field samples recognized the 24 and the 106 kd antigen bands with a few exceptions (Table 4-7). On the other hand, the 71 and the 81 kd antigens were recognized only by 15 and 11 of the 63 sera, respectively. This indicates that there were considerable variations in the *E. suis* antigen recognition by the field samples. As discussed above, antibodies produced in response to other infectious agents may cross-react to some extent with *E. suis* antigens, and these cross-reactions may contribute to this variation.

There were significant associations between the detections of the 37, 47, 71, 81 and 101 kd *E. suis* antigen bands in the western blots and the positive/negative results with the two ELISA's and DB (Table 4-8). The NetEs-Test's of the two ELISA's were positively associated with all 5 antigens, indicating that positive results of the NetEs-Test's with both ELISA's were strongly associated with the presence of *E. suis* specific antibodies in the sera. This was further confirmed by the observations reported in Table 4-6, that NetEs-Test of either ELISA exhibited a higher specificity for detecting sera that reacted with 6 or more of the selected 7 *E. suis* specific antigen bands, compared to CrudeEs-Test.

Even though there were significant associations between the detections of the 37, 47, 71, 81 and 101 kd *E. suis* antigen bands in the western blots and the positive/negative results with the two ELISA's and DB, the levels of association (contingency coefficients) were relatively low. The criteria used for selection of *E. suis* specific antigen bands was the band appearance in the autoradiograph of radiolabeled 100K antigen preparation (refer to Chapter 3). Since radiolabeling of *E. suis* was performed with *in vitro* culture for only 4 hours, it could be assumed that only proteins with sufficiently rapid turnover were radiolabeled effectively and proteins with a relative slow turnover were not. The 100K antigen used in the two ELISA's and DB contained the selected 7 *E. suis* antigens and possibly slow

turnover antigens. The results of the two ELISA's and DB reflected antibody reactions to both the rapid (the 7 *E. suis* antigens) and slow turnover antigens. Therefore, relatively low association levels of the two ELISA's and DB results and the reaction to the selected 7 *E. suis* antigen bands in western blots may be explained by the assumption that some of the two ELISA's and DB results are due to antibody reactions with slow turnover antigens.

CONCLUSION

Recently, *E. suis* DNA detection techniques have been developed using whole organism DNA hybridization (Oberst et al., 1990b) and recombinant DNA hybridization (Oberst et al., 1990a). These techniques detected *E. suis* specific DNA from the high salt lysate of whole blood samples, and apparently offer high sensitivity and specificity for detecting *E. suis* infection. Unfortunately at this time, a positive result with DNA hybridization indicates the presence of a current infection and does not detect past infections. These techniques could potentially lead to the development of a more powerful diagnostic technique using the DNA polymerase chain reaction (PCR), in which very low levels of infection could be detected by amplification of *E. suis* specific DNA. Even though PCR provides a more powerful diagnostic tool, serological diagnostic methods are still important for

conducting disease prevalence surveys. Unlike PCR, which requires sophisticated laboratory environment and relatively expensive equipment, ELISA can be performed at much less expensive and con be adapted for field use, allowing for quick assessment of antibody status.

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OVERALL CONCLUSION

In swine eperythrozoonosis, similar to other hemoparasitic diseases, the induction of autoantibodies against erythrocyte proteins is a major cause of confusion in the analysis of the organism proteins and results in poor specificity with serodiagnostic assays which use crude antigen preparations. The present study revealed a lack of specificity for detecting *E. suis* specific antibodies in the widely used IHA because of host derived impurities in the crude antigen preparation used in the test.

The *in vitro* culture system developed in this study facilitated the incorporation of a radiolabeled methionine by *E. suis*, thus enabling the analysis of organism specific proteins. However, to maximize the advantages of *in vitro* culture, a continuous culture system, as established in *Plasmodium falciparum*, needs to be developed for *E. suis*. Further screening of culture conditions, including optimum incubation temperature and pH, the supplementation of fetal pig serum, and additional understanding of the basic biology of *E. suis* will be indispensable for establishing a continuous *in vitro* culture system for *E. suis*. The glycolytic activity of *E. suis* observed *in vitro* suggests a

potentially significant role of hypoglycemia in the *in vivo* pathophysiology of swine eperythrozoonosis.

Among the several antigen preparations developed in this study, the 100K antigen preparation exhibited the greatest *E. suis* specificity. The specificity of the 100K antigen preparation, obtained by sequential differential centrifugation, was confirmed by the appearance of the *E. suis* antigen bands in western blots using only post infection sera and antigens prepared from parasitemic blood. Specificity was further confirmed by the band identity in the western blots using the 100K antigen and the autoradiograph of the radiolabeled *E. suis*.

The ProA- and IgG-ELISA, and DB developed in this study demonstrated high sensitivity for detecting antibodies in post-infection sera of experimentally infected pigs, regardless of using either CrudeEs-Test or NetEs-Test. However, impurities in the semi-purified 100K antigen still resulted in the detection of autoantibodies and isoerythrocytic antibodies. The NetEs-Test was developed to compensate for the detection of these antibodies and improved the detection of *E. suis* specific antibody responses. Indeed, subsequent western blot analysis that compared the results of the two ELISA's revealed that the NetEs-Test in either ProA- or IgG-ELISA was most significantly associated with the recognition of *E. suis* specific antigen bands in western blots.

Further purification of the 100K antigen preparation is recommended to eliminate interactions of host protein impurities in the antigen preparation with autoantibodies induced by *E. suis* infection. This would eliminate the necessity of using Es^+ and Es^- antigen preparations in the ELISA's or DB. The development of a rapid, sensitive and specific serodiagnostic assay could lead to a better understanding of the nature of disease including the true prevalence of *E. suis* infection and help to resolve the questionable associations of *E. suis* infection with impaired growth and reproductive performance. The characterization of *E. suis* specific antigens also could lead to the development of an effective vaccine.

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