Immunoenzymatic detection of antigens and antibodies in experimental porcine salmonellosis

by

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INTRODUCTION

Salmonella cholerae-suis var. kunzendorf is the major etiologic agent of swine salmonellosis in the United States today.[33] Modern intensive swine production practices have enhanced the occurrence of this disease[181] and economic loss associated with the infection of pigs necessitates the rapid and early detection of infected animals and carriers. The current methods for isolation and identification of Salmonellae from animal tissues require 2-4 days for definitive diagnosis. The process usually involves enrichment, isolation, biochemical, and serologic identification. The objective of this research was to investigate techniques wich could greatly decrease the time required to specifically identify Salmonellae. Assays were chosen which could easily be completed within one-half of a working day and within the capabilities of any diagnostic laboratory. Two enzyme-linked immunosorbent assays (ELISA) were developed: one to quantitate the antibody response of pigs experimentally infected with S. cholerae-suis var. kunzendorf, and another to detect the antigens of this organism in fresh tissues and enrichment broths from pigs similarly infected. Another assay for antigen was developed, the peroxidase-antiperoxidase immunoassay (PAP), which detects whole Salmonella cells in histologic sections. These techniques complemented each other on the type of information gained: ELISA quantified the antibody response in serum or antigen content of a

tissue; PAP demonstrated the presence of bacteria in association with surrounding host tissue. Therefore, this report has been divided into three sections:

1. Development of an Enzyme-linked Immunosorbent Assay to Detect the Antibody Response of Pigs Experimentally Infected with S. cholerae-suis var. kunzendorf.

2. Development of an Enzyme-linked Immunosorbent Assay to Detect S. cholerae-suis var. kunzendorf Antigens in Porcine Tissues.

3. Application of the Peroxidase-Antiperoxidase Immunoassay to the Identification of Salmonellae from Pure Culture and Animal Tissue.

LITERATURE REVIEW

Salmonella and Swine Disease

The success of *Salmonella* as a universal pathogen is exemplified by the observation that this organism has been isolated from nearly all vertebrate hosts from which it has been sought.[164] It is of interest in this review that one of the first associations of *Salmonella* with disease was a study implicating the bacterium as the causal agent of hog cholera, in which *Bacillus cholerae suis* was named.[139] The term "Salmonella" was coined by Lignieres in 1900 in honor of D. E. Salmon, the first Chief of the United States Bureau of Animal Industry.[91] Salmon's organism was accepted as the agent of hog cholera until the disease was reproduced in 1903 with a bacteria-free filtrate of body fluids taken from infected swine.[142] The confirmation of the viral etiology was made in 1904[48] and the importance of *Salmonella* as a swine pathogen was demoted to that of a secondary invader in pigs debilitated by hog cholera.[181] However, due to the recent eradication of hog cholera in North America, the bacterium is increasingly recognized as an important primary swine pathogen.[181]

Although over 50 *Salmonella* serotypes have been isolated from swine, *S. cholerae-suis* var. *kunzendorf* is the most common isolate in the U.S., followed by *S. derby* and *S. typhimurium*.[7] Also, *S.*

cholerae-suis var. kunzendorf is one of the 10 most commonly isolated Salmonella serotypes in animals.[33] The disease, also known as swine paratyphoid, manifests itself in one of two forms: an acute septicemic infection, or a subacute to chronic enteric form.[117] Swine of all ages are susceptible, but those a few weeks to a few months of age are most commonly affected.[7] Pigs surviving the acute disease continue to harbor the organism as carriers and the older the animal, the more likely it is to suffer from the chronic rather than the acute form of the disease.[7] Acutely affected animals suffer from weakness, loss of appetite, fever, and rapid respiration.[117] Diarrhea may or may not be present, but is a persistent symptom of the chronic disease, along with fever, rapid weight loss, and sometimes respiratory distress.

Porcine salmonellosis was first reproduced experimentally in 1927 by oral inoculation with S. suipestifer (cholerae-suis).[121] A detailed discussion of the sequential development of gross and microscopic lesions followed.[16] The disease syndrome caused by S. cholerae-suis var. kunzendorf is difficult to reproduce and results are inconsistent.[88] Subsequent studies on the pathology of the disease concentrated largely on the enteric lesions and results were conflicting.[49,71,146] Jubb and Kennedy[87] established clearly in 1963 that extra-alimentary lesions were a part of the syndrome and also gave the most complete description of the morphologic lesions up to that time. In 1966, Lawson and Dow were the first investigators to correlate pathological, clinical and bacteriological findings.[104] In

their examination of 96 whole pig carcasses diagnosed with S. cholerae-suis infection, they concluded the most frequent gross lesions to be: cyanosis of ears, limbs, and abdomen, splenomegaly, hepatomegaly, pulmonary haemorrhage, and, infrequently colitis. Other authors emphasize haemorrhage of the thoracic and visceral lymph nodes and petechiae in the renal cortex.[7,88,117] Gross intestinal lesions are less characteristic for salmonellosis, with progressive changes ranging from hyperemia and diffuse edema in the ileum, cecum, and colon to erosion of surface epithelium and appearance of "button ulcers" in the chronically ill pig.[88] Consistent histologic lesions in the Lawson and Dow study were: typhoid nodules in the liver containing aggregations of macrophages and occasional neutrophils or complete parenchymal necrosis, and, vascular lesions characterized by fibrinoid thrombi in the lung, kidney, and brain. Pneumonia in this condition is of the interstitial type because of the effect of the Salmonella on the alveolar vessels and is best observed in the diaphragmatic lobes.[88]

Information on the pathogenesis of S. cholerae-suis-induced lesions is incomplete. Much of this information comes from studies on serotypes other than S. cholerae-suis and in hosts other than swine, but generalizations can be made.[64,162,163] Accepted facts are that infections begin by ingestion and subsequent effects on the host are due to active bacterial aggression.[88,181] After ingestion of a small number of organisms, penetration occurs in the lymphoid tissues of the pharynx and the small intestine. Septicemia or transient bacteremia

results after a few days of residence and proliferation at these sites, followed by death of the animal or removal of organisms by fixed phagocytes in the spleen, liver, and bone marrow. Proliferation continues in these extravascular locations resulting in another round of bacteremia or secondary localization, during which time enteric localization occurs. Salmonella gain entry to the intestine via the liver and bile followed by mucosal invasion which is thought to be an absolute prerequisite for disease.[64] Pathogenicity of Salmonella, like other Enterobacteriaceae, is dependent upon the production of endotoxin, but it is not known which lesions are specifically endotoxic, immunologic, or ischemic in origin.[88,181] In summary, the pathogenesis of salmonellosis can be considered as two basic processes: systemic dissemination and local enteric replication. The outcome of the interaction between the host and Salmonella depends on the amount of time needed for the establishment of a critical population of bacteria.

Diagnosis and Control

Control of swine paratyphoid should center on the carrier pig. In contrast to S. typhimurium, S. cholerae-suis is a very infrequent isolate from pig feeds or nonporcine Salmonella reservoirs; therefore, the infected, shedding pig is considered as the major source of new infections.[181] The stress of transport and crowding on the way to slaughter has been shown to increase the amount of fecal shedding of

certain Salmonella serotypes by market swine.[182] But fecal shedding is unpredictable and the duration of shedding of S. cholerae-suis has not been studied. Furthermore, the organism is difficult to isolate from feces and is frequently present in mesenteric lymph nodes but absent from the intestinal lumen.[66,88,112] Serologic tests may detect previous exposure to Salmonella, but cannot be related to the carrier status or the probability of shedding.[66,181] This is because "O" and "H" agglutinins to common Salmonellae have been found in the sera of clinically normal pigs[111] and have been absent from others with apparent lesions of salmonellosis.[110]

Due to the lack of reliable, sensitive methods for the diagnosis of Salmonella infection in the live pig and the presence of symptomless carriers, consistent, definitive identification of S. cholerae-suis infection is limited to postmortem isolation of the organism from tissues. Isolation of S. cholerae-suis, as well as other Salmonellae from animal tissues, is a straightforward process involving enrichment, isolation, biochemical testing, and serologic identification. The most common enrichment medium utilized for this purpose is tetrathionate brilliant green broth[91,120] but several others are used according to preference and include Gram negative broth,[73] brilliant green MacConkey broth,[148,149] and selenite F broth.[105] Both tetrathionate and selenite broths have been reported as toxic for S. cholerae-suis,[105,148] yet both are recommended for general use in the diagnostic laboratory.[1,52] Toxicity of these media for S. cholerae-suis may explain the difficulty in recovering the

organism from feces,[52] where it is often present in low numbers as in carrier animals. For this reason many laboratories also incorporate direct plating of a sample onto a suitable selective agar such as brilliant green.[91,97,102] This medium is commonly used for isolation from enrichment broths, differentiating Salmonellae from other enteric bacteria on the basis of lactose fermentation. Isolated colonies can be subjected to a variety of biochemical tests, but a combination of 2 or 3 usually yields sufficient information for presumptive identification and subsequent agglutination tests. Kliger's iron agar,[52] lysine iron agar,[53] or triple sugar iron agar[160] and urea agar will differentiate Salmonellae from Citrobacter, Proteus, Enterobacter, Klebsiella, Shigella, and Escherichia coli.[40] Hydrogen sulfide production by S. cholerae-suis var. kunzendorf isolates is helpful in this process.

Serologic identification is required to differentiate S. cholerae-suis var. kunzendorf from other serotypes, most commonly S. typhimurium, S. derby, S. newport, and S. anatum (Table 1).[1] The rapid, accurate serological characterization of Salmonellae was made possible by White[179,180] and especially Kauffmann.[90,92,97] The Kauffmann-White Scheme for Salmonella is based on defined O (somatic), Vi (capsular), and H (flagellar) antigens.[98] Currently over 2000 serotypes are included in this scheme and reference laboratories routinely perform complete serologic and biochemical identification.[52] However, for general laboratory identification it is sufficient to

Table 1: Antigenic composition of Salmonellae commonly isolated from swine (in order of prevalence)[1]

		Antigen		
Serotype	Serogroup	O	H	
			Phase 1	Phase 2
S. cholerae-suis	C_1	6,7	[c]	1,5
S. derby	B	1,4,[5],12	i	1,2
S. typhimurium	B	1,4,[5],12	f,g	[1,2]
S. newport	C_2	6,8	e,h	1,2
S. anatum	E_1	3,10	e,h	1,6

Bracketed antigens may be lacking.
Underlined antigens are present only when organism is lysogenized by converting bacteriophage.

obtain only the serogroup designation, allowing the differentiation of most Salmonellae isolated from swine (Table 1). The antigenic formula for each serotype (e.g. S. cholerae-suis 6,7:c:1,5) represents the O antigens: the phase 1 H antigen(s): and the phase 2 H antigen(s), respectively. Certain serotypes (e.g. S. typhi) have an outermost polysaccharide layer, termed the Vi antigen. Those formulae with particular O antigens in common are collected into an O group and arranged alphabetically by H antigens within the group. The specificities of the O factors are determined by the composition and structure of the polysaccharides in the O-specific chains of lipopolysaccharide (LPS). A total of 18 different sugars (monosaccharides) have been identified in Salmonella LPS.[151] The organisms in a given culture may be entirely in one H antigen phase (monophasic culture) or can frequently give rise to mutants in the other phase (diphasic culture). This phase variation depends on reversible DNA transposition and can be selected for in vitro by growing the organisms in the presence of antibody to flagellar antigens of one phase.

Salmonella taxonomy is a matter of controversy. The Kauffmann-White Scheme, despite its complexity, is supported by the International Subcommittee on Enterobacteriaceae because of its widespread familiarity.[106] Other theories on the classification of this genus exist. Crosa et al.[42] suggest that the so-called "genus" Salmonella is, in fact, one species. This theory is based on the principle that bacteria which are related by 70% or more by DNA/DNA

hybridization experiments belong to the same "genospecies." Borman, Stuart, and Wheeler[18] proposed the subdivision of the genus into 3 species, S. cholerae-suis, "S. typhosa" (S. typhi), and "S. kauffmannii", the last to serve as a species for all the serological types. A similar proposal was made by Kauffmann and Edwards[99], but included "S. enterica" as an all-embracing species. The division of Salmonellae into 5 "subgenera" by Kauffmann[93,94,95,96] on the basis of biochemical characteristics corresponds closely to species or subspecies in other groups of bacteria,[106] and LeMinor, Rohde and Taylor[107] proposed the consideration of these "subgenera" as species. Most recently, LeMinor, Veron and Popoff[108] proposed nomenclatural changes for Salmonellae on the basis of numerical taxonomy and DNA relatedness studies: the genus should consist of a single species, S. cholerae-suis, and six subspecies based on Kauffmann's subgenera and additional biochemical and serological characteristics.

A fluorescent antibody (FA) test has been developed for the identification of Salmonellae from enrichment broths.[55] Although this could shorten the identification procedure significantly, use of the FA test is probably not widespread due to the need for special equipment and inherent nonspecific fluorescence. The isolation and identification procedures as outlined above require 2-4 days for definitive diagnosis. The use of direct plating can minimize this period, but should always be accompanied by enrichment.

The Enzyme-linked Immunosorbent Assay

General Information

The widespread application of the enzyme-linked immunosorbent assay (ELISA) has contributed significantly to the advancement of biomedical technology in the 1970s and 1980s. In general, this technique involves the detection of antigen or antibody by: 1) reaction of the unknown sample with a corresponding specific antibody or antigen bound to a solid phase, 2) application of a specific conjugate (enzyme-linked antiglobulin) which will localize the unknown, and 3) measurement of the reaction by the enzymatic degradation of substrate, indicated by a color reaction. The binding of two proteins, antibody and enzyme, for immunologic localization has infinite potential for the detection of biological molecules. But the whole sucess of this technique is dependent upon the gentle yet irreversible attachment of specific antibody to an enzyme whose activity is easily measured, without destruction of the antibody or enzyme activity. Such an accomplishment was not possible without preceeding and concomitant advances in immunochemistry, protein chemistry, and enzymology. Future improvements in the ELISA will depend on the laboratory worker's knowledge of both the vast number of applications of the assay reported in the literature of the last decade and the protein chemistry involved in the development of the test. The importance of the latter should not be ignored. Development of a successful, sensitive ELISA relies upon a clear

understanding of the immunochemical reaction necessary to measure minute quantities of an unknown antigen.

The underlying determinant of sensitivity in all immunoassays is the antibody affinity, or efficiency of binding, to antigen.[129] Antibody affinity can be affected by the labeling procedure, attachment to the solid support, and the individual animal that produced the antibody. Other factors influencing sensitivity are the denaturing effect of the solid support and the volume of the assay solution.

Development of the enzyme immunoassay commenced in 1942 with the fluorescein labeling of antibodies by Coons and coworkers.[38] Singer and Schick[144] were the first to couple two protein molecules for immunologic localization, ferritin and antibody, via a diisocyanate derivative. Subsequently, various coupling agents were used to attach enzymes to antibodies (conjugation). The use of water soluble carbodiimides,[6] cyanuric chloride,[4] and p,p'difluoro-m,m'dinitrophenyl sulfone[123,124] met with varying degrees of success in the formation of enzyme-labeled proteins used for the localization of antigens or antibodies within cells or for the characterization of antibodies after immunoelectrophoresis. However, the yield of conjugate was low (1-10% of original antibody added) or unstable.[5] The coupling of peroxidase to antibodies by glutaraldehyde[3] made enzyme-labeled localization practical for immunohistology. Coupling with glutaraldehyde can be accomplished by a one- or two-step procedure, both resulting in a good combination of yield and retention of

enzymatic and immunologic activity.[5] The simplicity and wide applicability of the one-step glutaraldehyde procedure currently make it one of the most popular coupling methods, along with the m-periodate technique.[122]

Choice of an appropriate enzyme-conjugate depends on the type of ELISA developed. Horseradish peroxidase is highly favored because of its low cost, ease of conjugation, and wide variety of substrates.[175] Alkaline phosphatase is also commonly used[101,174,175] and the less common enzymes include acetyl cholinesterase, catalase, cytochrome C, beta-D-galactosidase, glucoamylase, glucose oxidase, beta-D-glucoronidase, lactate dehydrogenase, lactoperoxidase, ribonuclease, and tyrosinase.[175] Suitability of an enzyme conjugate will depend on stability, availability, sensitivity, reactivity (which can be determined, for example, by size and penetrating ability) and the availability of a convenient substrate detector system.[184]

The great variety of reagents and the order in which they are added to a test system have resulted in many modifications of the original ELISA for quantifying immunoglobulin G pioneered by Engvall and Perlmann.[59] In general, an ELISA can be homogeneous or heterogeneous. Homogeneous enzyme immunoassays are restricted to the assay of small molecular weight substances (such as drugs) and require no steps to separate reacted from unreacted enzyme-labeled material.[136] Heterogeneous assays, which are the most common, require separation of unbound reagents by washing, and are suitable for the detection and measurement of large molecular weight substances (MW

over 10,000).[175]

The heterogeneous ELISA can be modified to detect antibody or antigen. Detection of antibody is commonly via the indirect method (Fig. 1): antigen is coated to the solid phase, followed by the test serum, the enzyme-labeled antiglobulin, and substrate.[175] Antigen detection can be accomplished by three methods or variations thereof (Fig. 2-4): competitive ELISA, double-antibody sandwich ELISA, and inhibition ELISA.[14]

ELISA results, based on the amount of substrate degraded, can be assessed visually or with the aid of a spectrophotometer. Visual readings are adequate when an end-point dilution of a sample is determined, but in many cases only a single dilution of serum or sample is tested, requiring spectrophotometric and statistical analysis.[14] Automatic reading devices are widely available and have made accurate assessment of ELISA results possible in most laboratories. Briefly, ELISA results can be expressed in the following ways:[175] 1) as "positive" or "negative", 2) as the absorbance value, 3) as a ratio of the absorbance value of the sample to the mean of a group of known negatives, 4) as an end-point titer, and 5) as a unit extrapolated from a standard curve of samples with known content.

The list of applications of ELISA in the serologic diagnosis of

Figure 1. Example of an indirect ELISA for antibody detection

Indirect ELISA

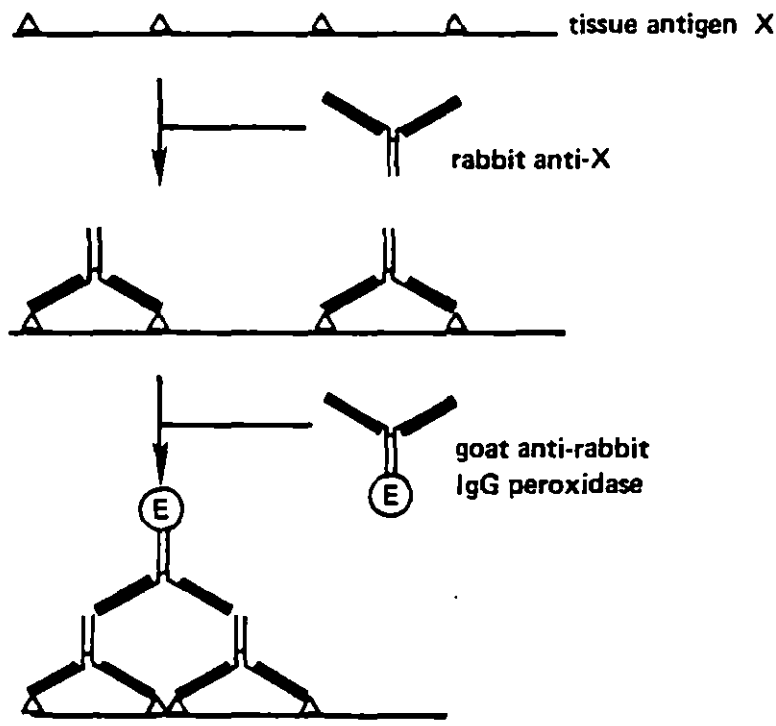
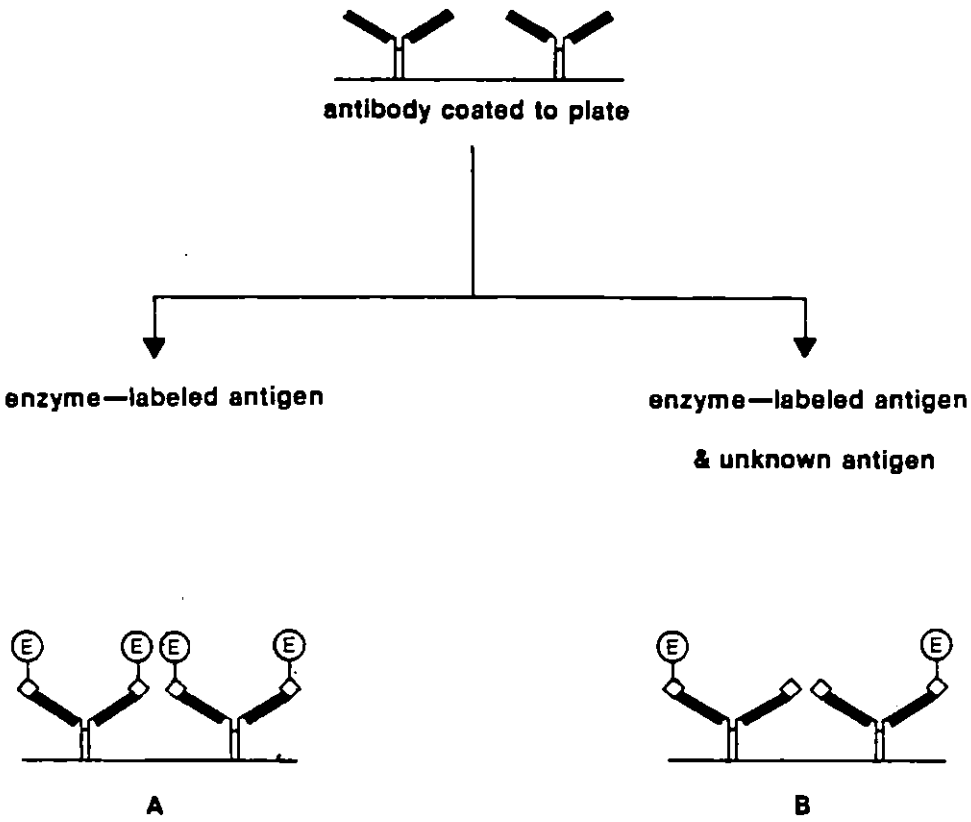


Figure 2. The competitive ELISA for measurement of antigen

Competitive ELISA



Substrate hydrolysis occurs with enzyme—labeled antigen.

Difference between A and B = amount unknown antigen.

Figure 3. Example of a double-antibody sandwich ELISA for antigen detection, modified because the second antibody added is not conjugated to enzyme. Enzyme-labeled antiglobulin is added in the next step

Modified Double-Antibody Sandwich ELISA

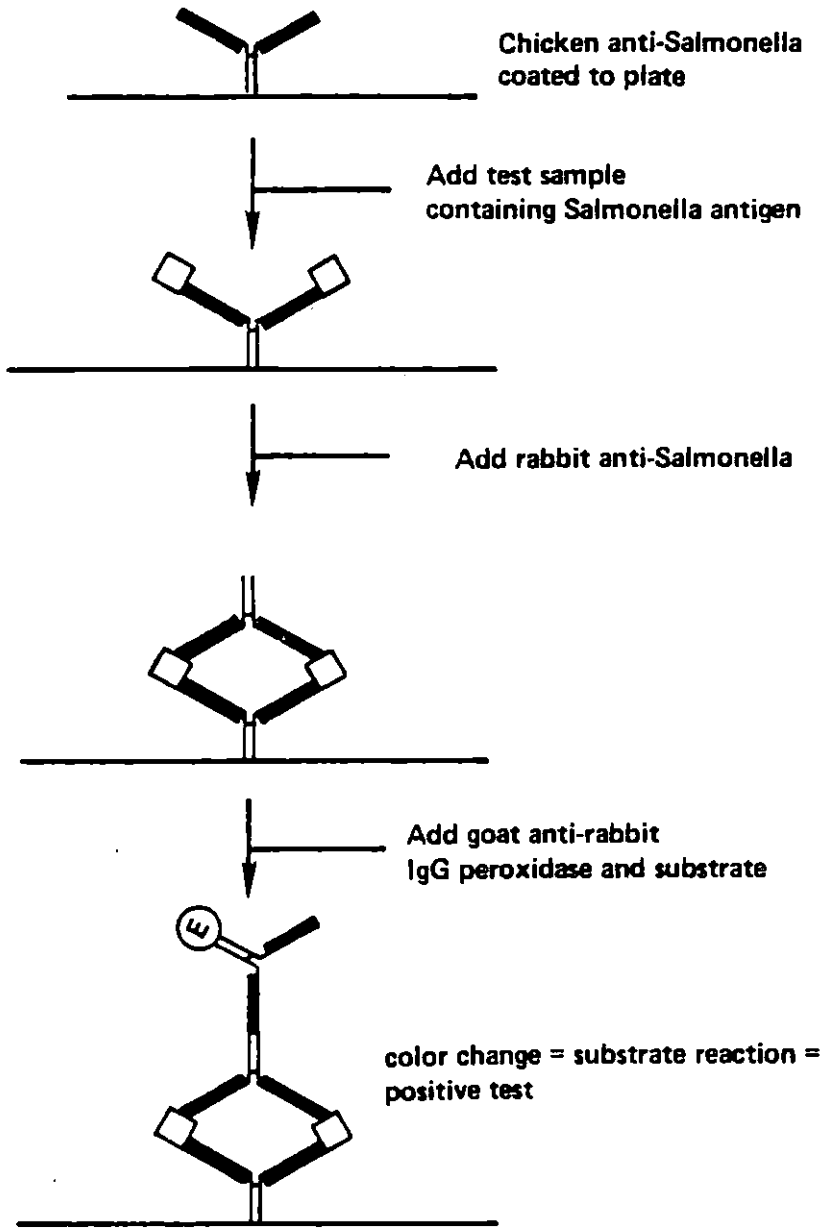
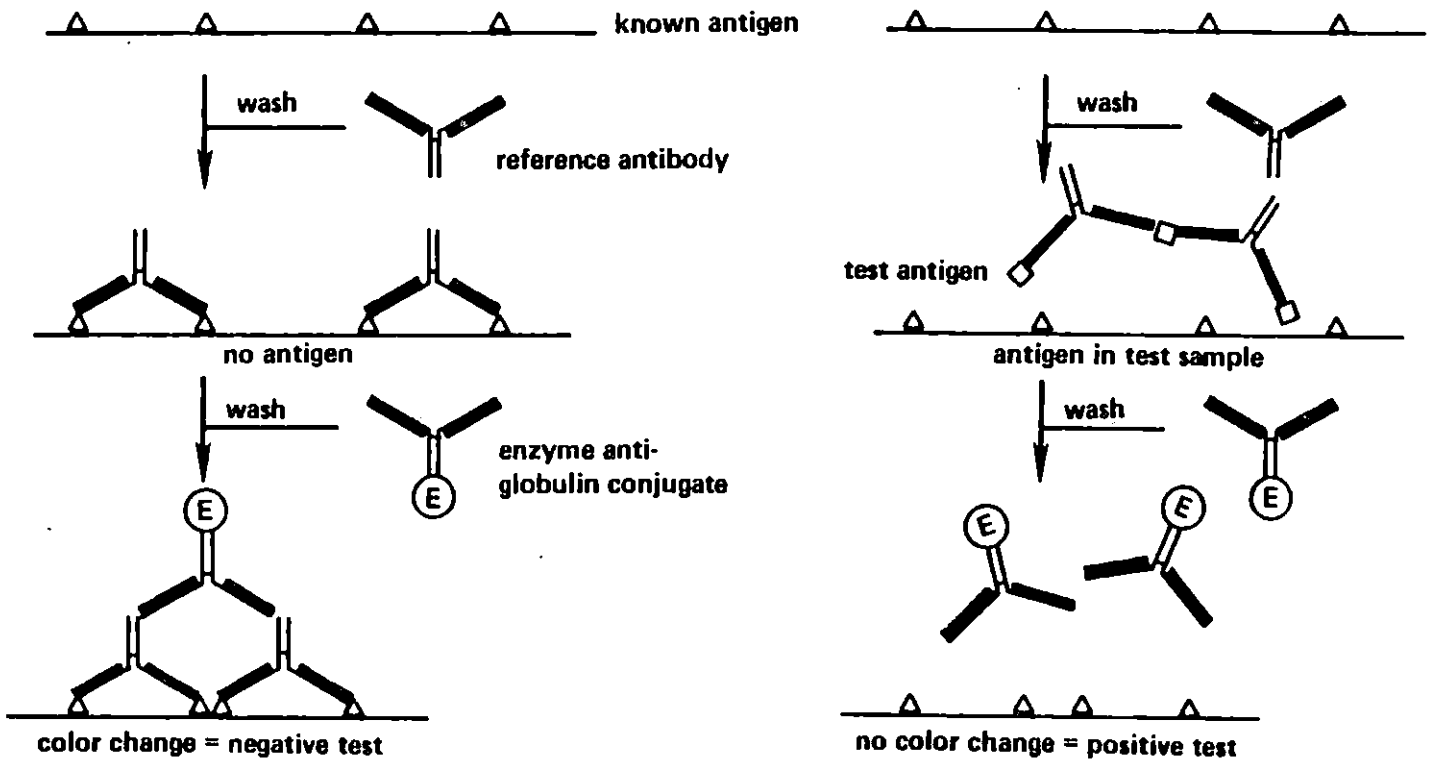


Figure 4. The inhibition ELISA for measurement of antigen

Inhibition ELISA



human and animal infectious disease is long.[8,15,17,20,24,39,47,56,65,67,72,75,76,81,82,83,85,126,127,132,133,141,166,176] But for the diagnostician, the technique is no panacea. The vast majority of the tests published never make the transition to a commercially available, standardized procedure. It is difficult for the veterinary or medical laboratory diagnostician to assess the usefulness of the techniques developed in the literature, and one must remember the conditions under which these tests are devised: well-controlled experimental situations with a limited number of subjects. Also, the testing of controls must be carefully scrutinized; positive, negative, and related positive samples are necessary. Still, several ELISAs have been developed into commercial kits for the detection of antibody or antigen,[130] and the potential of the technique for research use is unlimited.

Enzyme immunoassays for the detection of antigen are more difficult to develop. This is due to the need for a very high sensitivity in many cases and the presence of related antigens in many clinical samples. Sufficient sensitivity is particularly difficult to achieve with those microbial antigens which replicate intracellularly or that have a large number of immunologically distinct serotypes.[184] Yet the potential for sensitivity afforded by the ELISA in addition to its simplicity as compared to radioimmunoassay has resulted in the development of tests for the measurement of bacterial, viral, parasitic, and fungal antigens, as well as immunoglobulins, hormones, drugs, serum proteins, and tumor antigens.[184] The following bacterial

antigens have been detected by the ELISA: Legionella pneumophila,[10,169] Haemophilus influenza type b,[43,128,178] Streptococcus pneumoniae,[50,77] Escherichia coli K99,[55] E. coli colonization factor antigen I,[60] E. coli heat-labile enterotoxin,[186] Staphylococcus aureus enterotoxin,[125,157] Neisseria meningitidis,[13] Clostridium difficile heat-labile toxin,[185] and mycobacterial antigens.[168]

ELISA Diagnosis of Salmonellosis

The ELISA has been used for the detection of both antigens and antibodies in salmonellosis. The first study published was in 1972[30] in which an ELISA for the detection of antibodies against O antigens of different Salmonellae was found to be more sensitive than the Widal test (tube agglutination), indirect (passive) hemagglutination, and quantitative precipitation. The test, an indirect ELISA, was also highly specific and could differentiate IgG and IgM. The same investigators then applied the test to the diagnosis of typhoid and paratyphoid fever (S. paratyphi A, S. typhimurium, and S. typhi) in humans and found it to correlate significantly to the Widal reaction and to be more sensitive and reproducible.[31] Studies followed in the interest of developing polyvalent ELISAs for the diagnosis of a more general Salmonella infection.[109,145] Results were encouraging but the tests apparently have not met with widespread application. A great deal of clinical testing remains to be done. Investigators in

Sweden[89,161] have successfully applied ELISA to the serodiagnosis of S. enteritidis and S. typhimurium epidemics. Of a total 26 patients diagnosed to have S. enteritidis infection by positive fecal culture, 24 or 92% had elevated ELISA titers of antibody to lipopolysaccharide representative of Salmonella Group D. ELISA titers against S. typhimurium in infected patients were found to persist at least 3 years, to be highly specific, and to be most significant 18-24 days post-infection. These studies suggested a role for ELISA in epidemiological studies of salmonellosis and in the study of the carrier state. However, the established methods of fecal culturing will probably prevail for the diagnosis of Salmonella infection in humans. One study in the field of veterinary medicine[133] showed promise in the detection by ELISA of post-vaccinal antibody titers in cows given a killed S. typhimurium vaccine. The corresponding Widal reaction was not sensitive enough to detect these titers, and the ELISA results after vaccination correlated significantly to a decrease in clinical severity of disease in cows already infected and to the inability to recover the organism from feces. Other studies using ELISA for the serodiagnosis of animal salmonellosis are needed, especially for the tracking of carrier animals.

ELISAs for Salmonella antigen detection have been limited to food microbiology. Several tests have been devised[103,118,134,147] with sensitivities ranging from 10^5-10^6 bacteria/ml of sample or culture medium. Sensitivity levels in the 10^5/ml range were obtained after preenrichment for 4-46 hours.[103,118] The most recent investigations

involve the use of a monoclonal antibody to increase the sensitivity of the test. A competitive type solid-phase ELISA was devised with a limit of sensitivity of 10^3 bacteria/ml.[147] However, in this study, samples were concentrated in preparation for the test and this may have falsely enhanced the sensititivity of the assay, since other investigators utilizing the same monoclonal antibody[134] obtained a sensitivity of 10^6 bacteria/ml. Still, the monoclonal antibody technique can be useful because it reacts with a flagellar determinant common to many Salmonellae but not to other Enterobacteriaceae.[134,147] Also, the time required to perform the ELISA is still significantly less than the conventional enrichment culture procedure used in food microbiology. Therefore, ELISA for Salmonella antigen detection in food has been successfully developed and should be readily applied to the diagnosis of animal disease.

The Peroxidase-Antiperoxidase Test

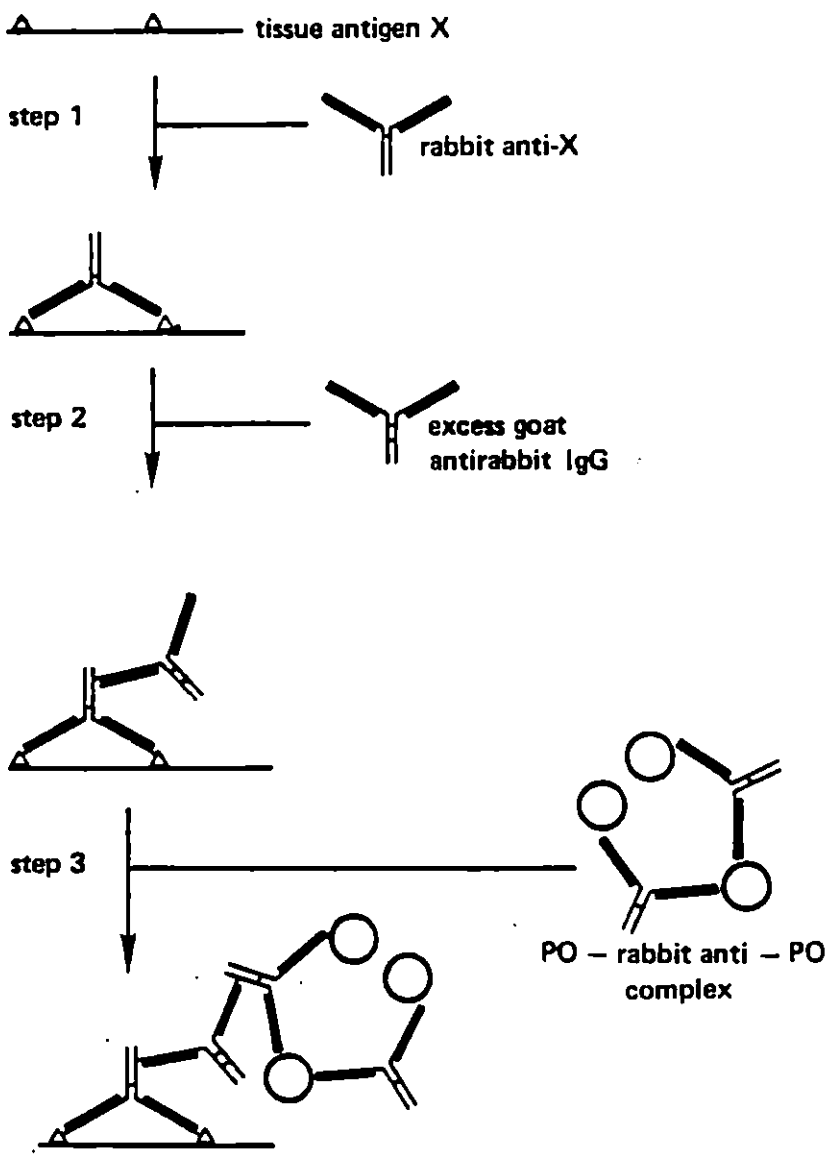
The peroxidase-antiperoxidase technique, also termed the unlabeled antibody enzyme method of immunohistology, arose as an attempt by Sternberger et al.[156] to increase the level of sensitivity afforded by other immunostaining procedures, such as by immunofluorescence and immunoferritin. Enzyme conjugates obtained by covalent labeling reactions did not increase sensitivities adequately. The immunohistochemical localization of antigen was shown to be intensified without the use of artificially conjugated antibodies by

utilizing anti-horseradish peroxidase antibody and horseradish peroxidase in separate steps of the staining procedure.[115,116,153,155] Sternberger et al.[156] modified the technique by preparing a soluble complex of horseradish peroxidase-antihorseradish peroxidase (PAP). This was accomplished by adding a moderate excess of peroxidase (antigen) to a washed precipitate of peroxidase-antiperoxidase which was then dissolved at low pH and temperature. Upon immediate neutralization there was reequilibration of PAP into soluble complexes of homogeneous composition. The resulting soluble PAP complex was separated from peroxidase by precipitation with ammonium sulfate. Analysis of the complex yielded data indicating that PAP was a pentameric complex consisting of two IgG and three peroxidase subunits, with a molecular weight of 410,000-432,000. Addition of anti-IgG to PAP precipitated 99.1% of enzymatic activity, indicating that the peroxidase in PAP was indeed bound and that there was no significant amount of free peroxidase. The homogeneity of the complex was supported by sedimentation and electron microscopic analysis.

The first application of the PAP method was for the identification of Treponema pallidum in experimentally infected rabbit tissue.[156] The procedure involves the sequential application of four basic reagents (Fig. 5): primary antibody directed to the antigen in question, secondary or link (anti-species) antibody, peroxidase-antiperoxidase of species origin identical to the primary antibody, and finally, hydrogen peroxide combined with a suitable chromogen such as 3,3'-diaminobenzidine tetrahydrochloride (DAB). The end product of

Figure 5. Example of the peroxidase-antiperoxidase test for the localization of antigen

PAP


tissue antigen X
step 1
rabbit anti-X
step 2
excess goat
antirabbit IgG
step 3
PO – rabbit anti – PO
complex

this enzyme-substrate reaction is insoluble and visible in light microscopy as a brown color or as electron-dense in electron microscopy when osmium tetroxide is the indicator.

Sensitivity of the PAP method has been demonstrated to be superior to that of immunofluorescence, enzyme histochemistry, and radioimmunoassay. T. pallidum was detected with dilutions of primary antisera 100 to 1000 times higher than those satisfactory for indirect immunofluorescence.[156] PAP was found to be at least twenty times more sensitive than the peroxidase labeled antibody sandwich method in the detection of human Kappa chains in formalin-fixed paraffin embedded tissues.[26] Adrenocorticotropin (ACTH) was detected in glutaraldehyde-fixed, Araldite-embedded rat pituitaries using a serum diluted 50 times higher using the PAP test than that used in radioimmunoassay.[119]

The principle of the PAP method avoids methodologic nonspecificity in contrast to labeled antibody methods where the reagents can contribute to background staining.[154] The link antiserum in the PAP system could conceivably react directly with tissue or with nonantibody components of the primary antiserum once they are attached to tissue. However, the free binding site of such a non-specifically bound link antibody can only react with a similar nonspecific component, and not with the purified PAP. In labeled antibody methods, in contrast, both the specific and nonspecific factors of the secondary antiserum are labeled, thus increasing the potential for nonspecific staining.

Another possibility for nonspecific staining in the PAP system is

binding to tissue by nonantibody components in the primary antiserum. But if these nonantibody components react with the link antiserum, PAP cannot be bound, thus the nonspecific reactions are not stained. Primary antibodies reacting nonspecifically with the tissue will be detected, however. This is minimized by pretreating sections with normal serum from the same species donating the link antiserum.[26,154,156] Also, concentrations of primary antiserum above 1:50 should be avoided in the PAP system. These may yield background staining due to cross-reacting antibodies or nonspecific binding of immunoglobulin in the primary antiserum.

Labeled antibody methods require the mildest possible fixation of tissues in order to preserve a maximum of antigenic reactivity. But because of the sensitivity of the PAP method, one must no longer preserve most determinants of an antigen.[154] Since the method is 100-1000 times more sensitive than immunofluorescence, 99 out of 100 antigenic determinants can be destroyed without loss of detection. This is equivalent to use of a 100-fold lesser dilution of the serum on frozen tissue in which all the determinants have been retained. Fixatives which cause extensive destruction can be used with most antigens in the PAP system since it employs highly diluted primary antisera. Those fixatives which best preserve structural integrity are optimal since it is apparent that loss of antigen from tissue during embedding is minimized with structural preservation. Halmi and Duello[74] found that routinely processed tissues could be examined decades after storage in paraffin, and even old slides stained by

hematoxylin and eosin could be rediagnosed after destaining and PAP processing.

Sternberger suggested the use of a single fixative when initiating a PAP test.[154] If the primary antiserum were questionable, a dilution of 1:1000 was to be used. A 1:100 dilution was included if the serum were suspected of extremely low antibody contents; however, a block titration of the antiserum was not necessary. Only if staining were not observed with these procedures, was it worthwhile to explore other approaches such as frozen sections or fixed vibratome sections. Vibratome sections may be superior to paraffin sections in the detection of lipid-soluble antigens since the lipids are extracted in the solvents used for embedding.

Whole antisera rather than immunoglobulin fractions were recommended in the PAP method as primary and link antisera. If the antisera were to be purified, solid-phase immunoabsorption of nonspecific antibodies was a better approach than purification of the specific antibodies, since specifically purified antibodies often represented the fraction possessing the lowest affinity and lowest specificity.

Endogenous peroxidase activity was apparently not destroyed by paraffin embedding. Streefkerk[159] stopped this activity by exposing deparaffinized sections to a 0.5% solution of hydrogen peroxide in methanol for 30 min. Several other methods for inactivating endogenous peroxidase have been found appropriate by other investigators. These include methanol and nitroferricyanide,[158]

sodium azide and hydrogen peroxide,[11] periodic acid and sodium borohydride,[86] acidified methanol,[61] acid alcohol or hydrazine,[27] and pepsin.[131]

Documented applications of the PAP technique include: detection of enzymes, polypeptide and steroid hormones, immunoglobulins, oncodevelopmental antigens, and viral antigens.[44] Interestingly, the technique has not been used to any great extent in bacterial identification since Sternberger's original article.[156] Short and Walker followed spore formation in Bacillus cereus via PAP and electron microscopy.[143] Woodland et al.[183] found PAP and immunofluorescence 50-100% more sensitive than the Giemsa stain in detecting Chlamydia psittaci in feline conjunctival scrapings and in cell culture. But the immunohistochemical localization of bacteria has been more commonly accomplished via direct and indirect immunoperoxidase techniques.[12,23,28,34,41,80,170,165] This was likely due to several factors: fewer incubation steps in direct and indirect immunoperoxidase tests and therefore less time required for the procedure, greater availability of commercially labeled enzymes as compared to PAP (although PAP is now becoming widely available), and direct and indirect tests may be easier and more rapidly developed. But the advantages of the PAP technique demonstrate a potential for use in bacterial identification. In summary, the advantages of the PAP technique include greater sensitivity due to the use of unlabeled antibody, ability to use a wide variety of tissue fixatives,

availability of commercially prepared reagents (secondary antiserum and PAP), and rapidity of the test (2-4 hours).

PART I.

DEVELOPMENT OF AN INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY TO DETECT THE ANTIBODY RESPONSE OF PIGS EXPERIMENTALLY INFECTED WITH *SALMONELLA CHOLERAE-SUIS* VAR. *KUNZENDORF*

Summary

An indirect enzyme-linked immunosorbent assay (ELISA) was developed to detect the antibody response of pigs experimentally infected with *Salmonella cholerae-suis* var. *kunzendorf*. The immunosuppressant cyclophosphamide (CY) was administered to 2 of 4 groups of pigs prior to or at the time of infection. The antigen preparation used in the ELISA was a freeze-thaw extract (FT) of the organisms which was found to be a preferable test antigen to lipopolysaccharide or O-antigen. All pigs were found to have elevated antibody titers to *S. cholerae-suis* FT by 2 weeks post-infection (p.i.). Those pigs not receiving CY reached the highest mean antibody titer of the 4 groups by 4 weeks p.i., while the only group demonstrating a detectable response by 1 week p.i. received bacteria and CY simultaneously. End-point titers obtained by ELISA were comparable to indirect hemagglutination titers from previous studies employing pigs experimentally infected with *S. cholerae-suis*, but the ELISA was easier to develop and interpret.

Introduction

The nature of the immune response of swine to salmonellosis caused by Salmonella cholerae-suis var. kunzendorf is poorly understood. Immunity to Salmonellae in general is thought to be primarily cell-mediated[36] although the humoral response is believed to play a role in limiting the infection. This problem was investigated in our laboratory utilizing the immunosuppressant cyclophosphamide (CY).[68] The major effect of this drug is on all rapidly dividing cell types such as B cells[114] thereby suppressing humoral immunity. The purpose of this study was to develop an enzyme-linked immunosorbent assay (ELISA) to measure the antibody response of swine experimentally infected with S. cholerae-suis and CY.

The indirect ELISA for antibody detection has been found useful by other workers in the serologic diagnosis of human and animal salmonellosis.[30,31,89,109,133,145,161] Purified lipopolysaccharide (LPS) was commonly employed as antigen. The present study compared LPS and two other antigen preparations for efficacy in the indirect ELISA in addition to the determination of antibody titers from 18 pigs injected with S. cholerae-suis and CY.

Materials and Methods

Rabbit serum

Anti-*S. cholerae-suis* serum was produced in rabbits for use in the development of subsequent enzyme immunoassays. Three New Zealand White rabbits were given serial intravenous or intramuscular injections of a heat-killed bacterin prepared in the following manner. Four of 72 *S. cholerae-suis* var. *kunzendorf* field strains were randomly selected from aliquots stored at -70C, pooled, and inoculated into trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD). After overnight incubation at 37C, the broth was autoclaved for 15 min, centrifuged and the bacteria washed in phosphate-buffered saline (PBS, pH 7.2), and diluted in PBS to a density of McFarland Tube #4. For intramuscular injections, 12% aluminum hydroxide was added as an adjuvant. A pre-injection serum sample was obtained from each rabbit for negative controls and hyperimmune sera were obtained 3 weeks after the first injection. Sera were tested for O titers using tube agglutination; the serum with the highest titer (1:1280) was used as a positive control in the ELISA.

Pig serum

A large number of reference porcine sera were available from previous experiments in the laboratory. The anti-*S. cholerae-suis* var. *kunzendorf* titers of these sera had been determined previously by indirect hemagglutination (IHA, determined by agglutination of sheep

erythrocytes passively coated with S. cholerae-suis LPS). Samples were selected for the titration of the ELISA which were negative, weakly positive, and strongly positive by IHA.

Unknown sera were obtained from 20 Yorkshire cross pigs weighing approximately 6 kg at the initiation of infection. These pigs were randomly divided into 4 groups of 5 pigs and housed in isolation units with concrete floors. Two of the 4 groups of pigs were given 20 mg/kg cyclophosphamide (CY) injected subcutaneously on days 0, 2, and 4 and received 3×10^6 S. cholerae-suis var. kunzendorf intramuscularly (IM) at different times thereafter. The dose of CY was chosen on the basis of data from previous investigations[114] and the overall effects of CY on the immune response of pigs to S. cholerae-suis var. kunzendorf was the subject of another study in the laboratory.[68] The remaining 2 groups of pigs received only S. cholerae-suis challenge (3×10^6 IM). The experimental design is summarized in Table 1.

Table 1: Experimental design of study involving pigs injected with S. cholerae-suis var. kunzendorf and cyclophosphamide (CY). n = number of pigs in each group

Group	n	CY	infected
1	5	-	+
2	5[a]	+	+(day 0)[b]
3	5	+	+(day 4)
4	5	-	+

[a]One pig in group 2 died on day 7, another on day 11, resulting in 3 pigs remaining for serum collection.

[b]Day 0 was the first day of CY administration or the day of infection if CY was not given.

Antigens

Three types of preparations from S. cholerae-suis var. kunzendorf were tested as coating antigens in a modified indirect ELISA. The antigen preparations tested were freeze-thaw (FT), lipopolysaccharide (LPS), and O-antigen. They were prepared as described below from the four field strains of S. cholerae-suis var. kunzendorf used previously for production of rabbit antiserum. These strains were pooled and grown in TSB at 37C for 24 hours.

FT. Broth cultures were pooled and subjected to 20 freeze-thaw cycles. The broth was then centrifuged to pellet bacteria and the supernatant filtered through a 0.45 µm Millipore filter (Millipore Corporation, Bedford, MA). After the supernatant was checked for sterility by plating on blood agar, the protein content of the

preparation was measured by the Bio-Rad Protein Assay (Bio-Rad Laboratories, New York, NY). The antigen was aliquoted and stored at -20C.

LPS. S. cholerae-suis var. kunzendorf LPS was prepared by the phenol-water extraction method of Westphal et al.[177]

O-antigen. O-antigen was prepared according to the method of the U.S. Public Health Service.[171] Four strains of S. cholerae-suis var. kunzendorf were grown as above. The broth was centrifuged and the bacteria were washed once in saline and resuspended in saline. An equal volume of 95% ethanol was added gradually with stirring and the mixture allowed to stand at room temperature overnight. One volume of saline was added for each 2 volumes of suspension. Finally, phenol was added to a concentration of 0.5% and the antigen stored at 5C.

Reagents for ELISA

All reagents, with the exception of substrate diluent and stock substrate solution, were freshly prepared every two weeks.

Wash Buffer (PBS-Tween).[168]

NaCl	29.0 g
Na_2HPO_4	2.3 g
NaH_2PO_4	0.2 g
Tween 80	5.0 ml
Deionized H_2O	1000.0 ml
pH adjusted to	7.5

Serum diluent.[168] Same as wash buffer except that twice the

concentration of Tween 80 was used.

Coating buffer.[2] 0.1M $NaCO_3$, pH adjusted to 9.6.

Carbodiimide solution.[2] 0.2mg/ml carbodiimide (Cyanamide, Sigma Chemical Co., St. Louis, MO) in 0.1M $NaCO_3$, pH adjusted to 9.6.

PBS.

Solution A: 0.01M KH_2PO_4 0.15M NaCl

Solution B: 0.01M Na_2HPO_4 0.15M NaCl

Adjust Solution B to pH 7.5 with Solution A.

Ammonium chloride solution.[2] 0.1M NH_4Cl.

Peroxidase conjugate diluent. 0.1% bovine serum albumin in PBS (Kirkegaard-Perry Laboratories, Inc., Gaithersburg, MD).

Substrate diluent.[150] 0.05M citric acid, pH adjusted to 4.0 using 5M NaOH. Stored at 4C.

Stock substrate solution.[150] 40mM ABTS (2,2'-azino-di-3-ethylbenzthiazoline sulfonic acid, Sigma) or 0.5487g/25 ml distilled water. Stored at 4C in a sealed, amber bottle.

Working substrate solution.[150] Prepare no more than 5 min before use.

25ml substrate diluent

100µl 3% H_2O_2

125µl stock ABTS

Selection of antigen and antigen coating method

The FT, LPS, and O-antigen preparations were subjected to preliminary testing with rabbit antiserum in a modified indirect ELISA to determine which reacted to the greatest extent. In addition, an O-antigen preparation from S. dublin was tested. Twofold dilutions of each antigen in coating buffer were made from 1:20 to 1:40,960. Fifty microliters per well of each dilution were added from top to bottom in a Dynatech Immulon I 96-well flat-bottomed polystyrene microplate (Dynatech Laboratories, Inc., Alexandria, VA). Two plates were used per antigen, one for the coating method utilizing carbodiimide as a coupling agent[2] and another for passive coating at alkaline pH and incubation at 37C overnight. For coating with carbodiimide, 50 microliters of carbodiimide solution were added to each well and plates were incubated at 4C overnight. After incubation, plates were washed three times in PBS. Fifty microliters of ammonium chloride solution were then added to each well and plates incubated at room temperature on a shaker (Arthur H. Thomas Co., Philadelphia, PA) for 30 min. Plates subjected to passive antigen coating were washed 3 times in PBS. Thereafter all plates were treated alike.

Plates were first coated with antigen by one of two alternative methods described above. Fifty microliters of rabbit antiserum against S. cholerae-suis var. kunzendorf diluted twofold from 1:100 to 1:16,000 were added to each well so that a block titration resulted: serum was diluted from top to bottom and antigen from left to right. Serum diluent only was added to the bottom row. Antiserum was incubated for 30 min on a shaker as before, followed by 3 washes in PBS-Tween. Thereafter 50 microliters of goat anti-rabbit serum (Polysciences, Inc., Warrington, PA) diluted 1:50 were added per well for 30 min as before, followed by 3 washes in PBS-Tween. Rabbit peroxidase-antiperoxidase (PAP, Polysciences) diluted 1:100 was added in 50 microliter quantities per well and incubated and washed as above. One hundred microliters of substrate (ABTS) were added per well and incubated for 30 min at room temperature without agitation. Color reactions were read visually and graded from 0 (colorless) to 4 (dark green).

Titration of FT antigen with porcine antiserum

The FT antigen was chosen as the best coating antigen for the ELISA (see Results, Fig. 5). In order to determine the optimum dilution of FT for use in the indirect ELISA, twofold dilutions of the antigen from 1:100 to 1:204,800 were added from left to right in the microplate. Antigen coating was performed as described previously utilizing carbodiimide. Three porcine sera were selected on the basis of indirect hemagglutination (IHA) titers so that a strong positive

serum (1:1024), weak positive (1:8) and a negative serum (no IHA titer) were included. Each serum was diluted 1:100 and 100 microliters were added per well to two rows of the plate. Plates were incubated for 30 min on a shaker at room temperature and then washed eight times in PBS-Tween. The conjugate, rabbit anti-porcine peroxidase (Cappel Laboratories, Cochranville, PA), was added in 100 microliter quantities per well and incubated and washed as above. One hundred microliters of substrate were added per well and plates allowed to stand at room temperature for 60 min. Optical densities were measured with a Dynatech MicroELISA Reader (Dynatech) equipped with a 405 nm bandpass filter.

The following controls with each ELISA plate run included: one row of serum diluent containing no anti-S. cholerae-suis serum, 2 wells containing substrate only, and 2 wells containing 100 microliters each substrate and conjugate added simultaneously.

Titration of porcine antiserum

Using the optimum dilution of FT antigen obtained in the previous step (see Results, Fig. 6), the porcine anti-S. cholerae-suis var. kunzendorf serum was titrated. The indirect ELISA was performed as above, except that antigen concentration was held constant while antiserum was diluted twofold from 1:20 to 1:40,960. Antiglobulin conjugate was again used at a 1:200 dilution.

Titration of antiglobulin conjugate

The rabbit anti-porcine peroxidase conjugate was titrated using the optimum dilutions of antigen and antiserum. The twofold dilutions of conjugate ranged from 1:200 to 1:1600. Indirect ELISA procedures were as described above.

Testing of unknown sera

Using the optimum test conditions as determined by the above methods (see Results, Fig. 5-7), the 76 unknown porcine sera were tested for anti-S. cholerae-suis antibody levels. Serial twofold dilutions of each serum were made and tested in duplicate.

Results

Optimal test conditions

All 3 antigen preparations, FT, LPS, and O-antigen, reacted similarly in the modified indirect ELISA (Fig. 1-3). S. dublin O-antigen reacted to a much lesser extent than the S. cholerae-suis antigens (Fig. 4). FT was chosen for subsequent ELISA tests because it was somewhat more detectable at higher antibody dilutions (e.g. 1:1600), probably contained a wider variety of antigens than the LPS, and contained a measurable amount of protein. Apparently the ethanol or phenol interfered with measurement of protein in the O-antigen. FT was found to contain 1.92 mg/ml protein by the Bio-Rad Assay.

Carbodiimide was found to greatly enhance antigen binding to the

plate (Fig. 1-3) and was used in all subsequent ELISAs developed.

Results from reagent titrations yielded curves in which the optimum dilution of each reagent was determined as the point with the highest specific reaction (with positive serum) and the lowest nonspecific reaction (with negative serum). From these determinations, it was found that the optimal antigen dilution was 1:500 (Fig. 5), antibody 1:80 (Fig 6), and conjugate 1:400 (Fig 7).

Unknown sera

Optical density means and standard errors of means are displayed in Fig. 8-10 for the 4 groups of pigs. Results were interpreted as end-point titers according to the method of Voller et al.[175] End-point titers were recorded as the highest serum dilutions with absorbance values significantly higher than the highest pre-infection serum value within the group. For example, in Figure 8, all negative serum O.D. values were at or below 0.142± 0.015. At two weeks post-infection, the highest dilution with an O.D. reading above this value was 1:160, with an O.D. reading of 0.19± 0.02. These titers are summarized in Table 2. Using this method of interpretation, all 4 groups of pigs demonstrated an increase in mean antibody response by 2 weeks post-infection (Fig. 8-10). Group 2, which received CY and S. cholerae-suis simultaneously, showed an antibody response by 1 week post-infection (Fig. 9). Those pigs not receiving CY (Fig. 8) reached the highest end-point titer of the 4 groups by 4 weeks post-infection.

Discussion

The ELISA developed in this study proved to be very suitable for the detection of porcine antibody to S. cholerae-suis var. kunzendorf in an experimental situation. Although serology is not normally used for the diagnosis of porcine salmonellosis, it allowed a comparison of the immune response of pigs differentially treated with an immunosuppressant. Results in Table 2 suggest that CY at the 20mg/kg dose was not sufficient to depress the B cell population to the extent that antibody formation was precluded. Those pigs not receiving CY attained the highest titer by 4 weeks post-infection, but the only group demonstrating a response by 1 week was that which was given CY and S. cholerae-suis simultaneously (Group 2).

The wide availability of equipment, reagents, and procedural guidelines for the ELISA make it a convenient test to develop. Standardization and control is straightforward to achieve in lieu of the ability to set negative values below a determined absorbance reading. Probably the greatest problem associated with ELISA is its day to day variability. This exemplifies the need for samples from one animal to be run all in one day and on one plate if possible; it is also desirable to have the same technician perform the assay from day to day if related samples are tested. Nevertheless, the ELISA is superior to other tests used in the serologic diagnosis of salmonellosis, such as indirect hemagglutination (IHA) and agglutinin titration (Widal). Previous studies in our laboratory utilizing IHA

for the detection of anti-S. cholerae-suis antibody have resulted in analogous yet somewhat lower end-point titers in pigs similarly infected.[69] One investigation[32] demonstrated close agreement between ELISA and IHA results for detection of antibody to cytomegalovirus, while another[30] found ELISA titers 10 to 100 times higher than corresponding IHA and Widal Salmonella titers. The same investigators later emphasized the preferential detection of IgM class antibodies by IHA and Widal whereas ELISA can pick up IgG and IgM with about equal sensitivity.[29] In addition, IHA requires more time and is less convenient when one considers the repeated absorptions of sera with red blood cells and the need for fresh, washed red blood cells for each test. Antiglobulin added after the IHA can increase sensitivity but specificity is also decreased in some cases and about twice the amount of time is required to perform the test. The day to day variability of the ELISA is probably no more significant than the often subjective reading of IHA which can change the titer from one dilution to the next higher or lower one. This variability becomes less significant in the ELISA when the number of samples increases and can then be statistically corrected.[150] Agglutinin titration can give an estimation of the degree of a hyperimmune response but has lost much of its former importance in the diagnosis of human typhoid and paratyphoid fevers because of problems in interpretation and the lack of generally accepted standards of procedure.[63]

The enhancement of antigen coating in the ELISA via carbodiimide has been observed by other authors.[35,167] The specific mechanism of

the effect of carbodiimide is not well understood but this compound has been used for the covalent coupling of proteins and as a cross-linking fixative in histochemistry.[9,19] Polymerization via carbodiimide occurs via the activation of carboxyl groups which then cross-link adjacent amino groups through amide bonds.[152] Therefore activated carboxyl groups may more easily bind to charged sites on the polystyrene surface. Residual active charge present on the plate is suggested to be neutralized by the addition of ammonium chloride, applied after the overnight coating of antigen.[2]

Visual readings in Fig. 1-4 did not always follow classical checkerboard titration patterns. Some "prozone" effects were observed, especially at higher antibody dilutions, which reflected the optimum combination of antigen and antibody at particular dilutions. Also, visual readings are less accurate than spectrophotometric readings, but were adequate for this part of the development of the test to compare the 4 antigen preparations.

Specificity of the ELISA developed was favorable. Very low readings were obtained with hyperimmune rabbit serum against S. dublin (Fig. 4), and pre-infection values in the pig system remained low, always below O.D. 0.2. This specificity is especially worthwhile in view of the ubiquity of Salmonellae.

Table 2. ELISA end-point serum titers of pigs experimentally infected with S. cholerae-suis var. kunzendorf. These titers were obtained by analysis of Fig. 8-10

Group	1 week p.i.	2 weeks p.i.	4 weeks p.i.
1 and 4	0	1:160	1:320
2	1:40	1:80	1:80
3	0	1:160	1:160

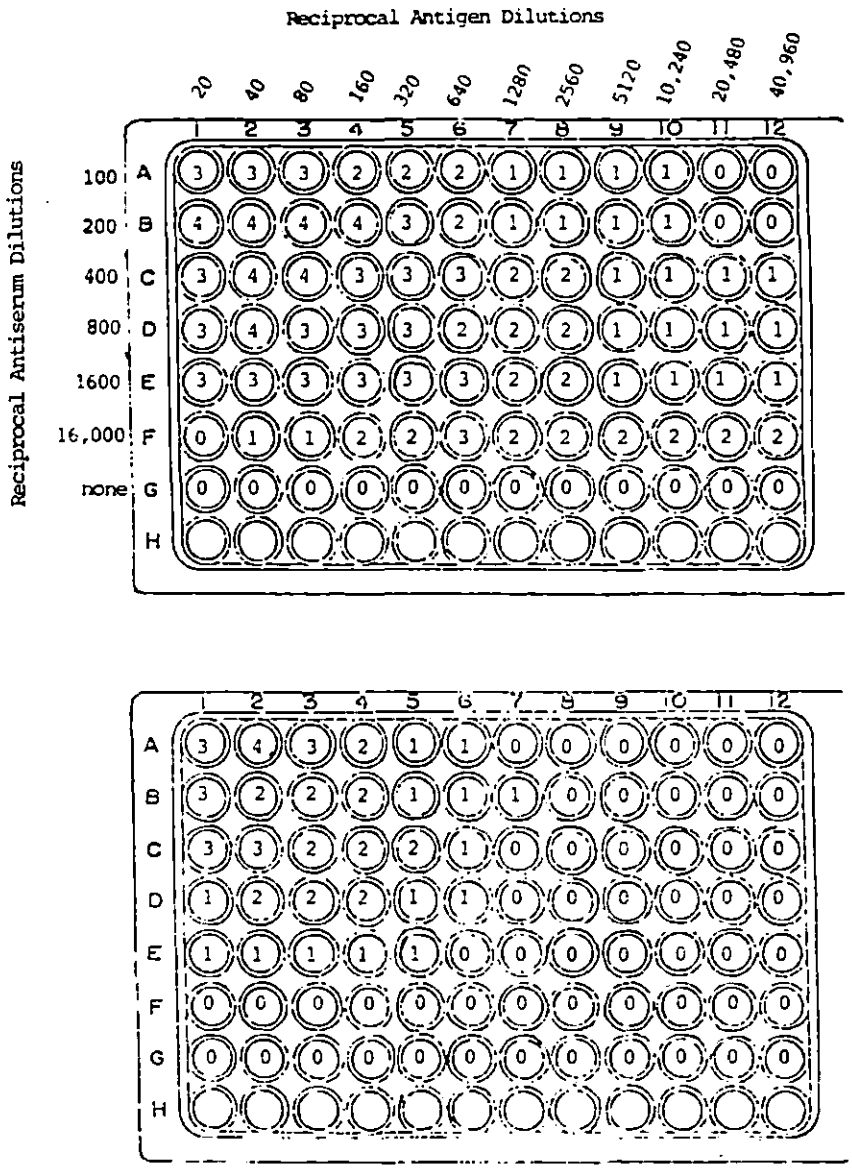


Figure 1. Modified indirect ELISA using S. cholerae-suis freeze-thaw (FT) antigen as coating antigen. Numbers indicate visual color readings ranging from 0 (negative) to 4 (most intense). A. Carbodiimide-activated coating of antigen. B. Passive coating.

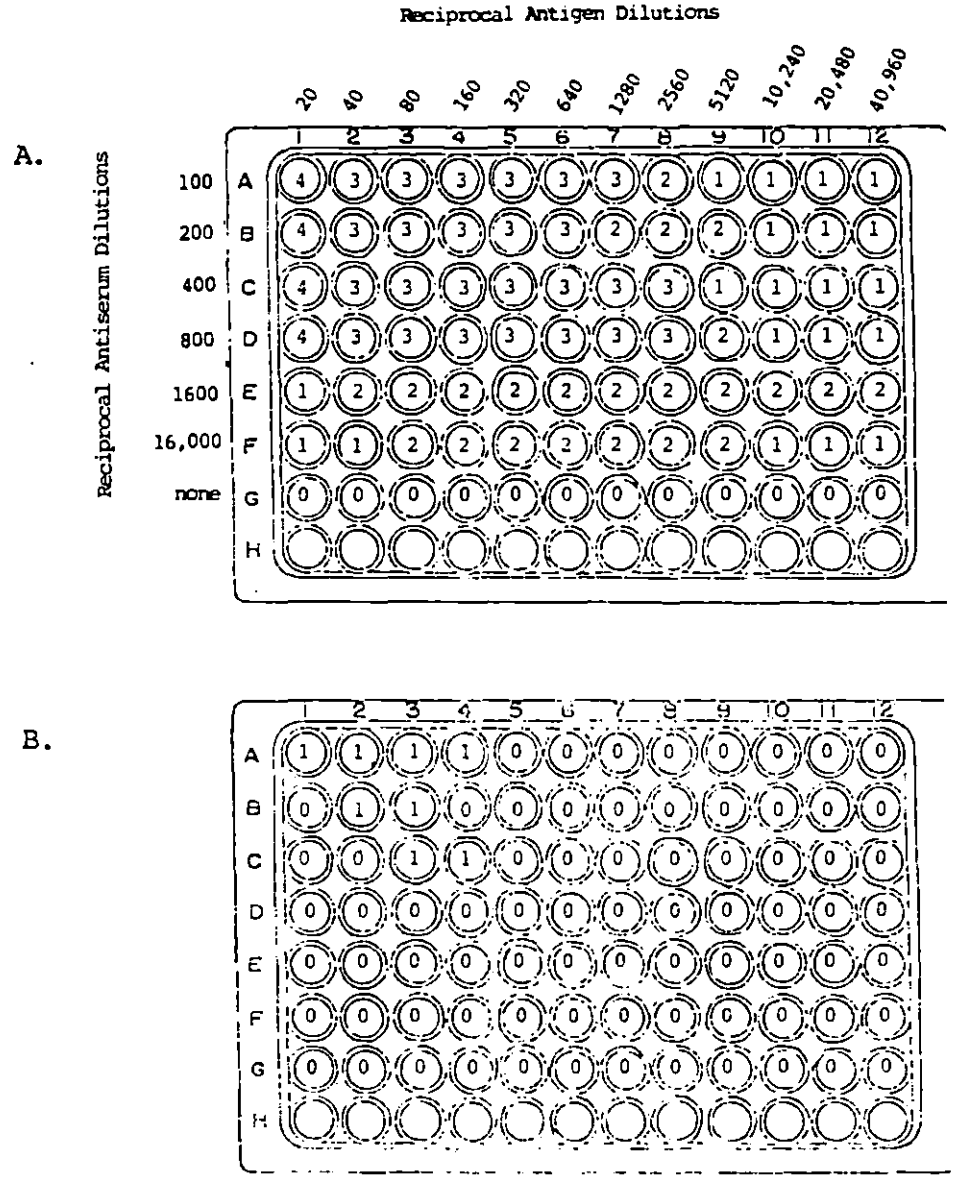


Figure 2. Modified indirect ELISA using S. cholerae-suis LPS as coating antigen. Numbers indicate visual color readings ranging from 0 (negative) to 4 (most intense). A. Carbodiimide-activated coating of antigen. B. Passive coating.

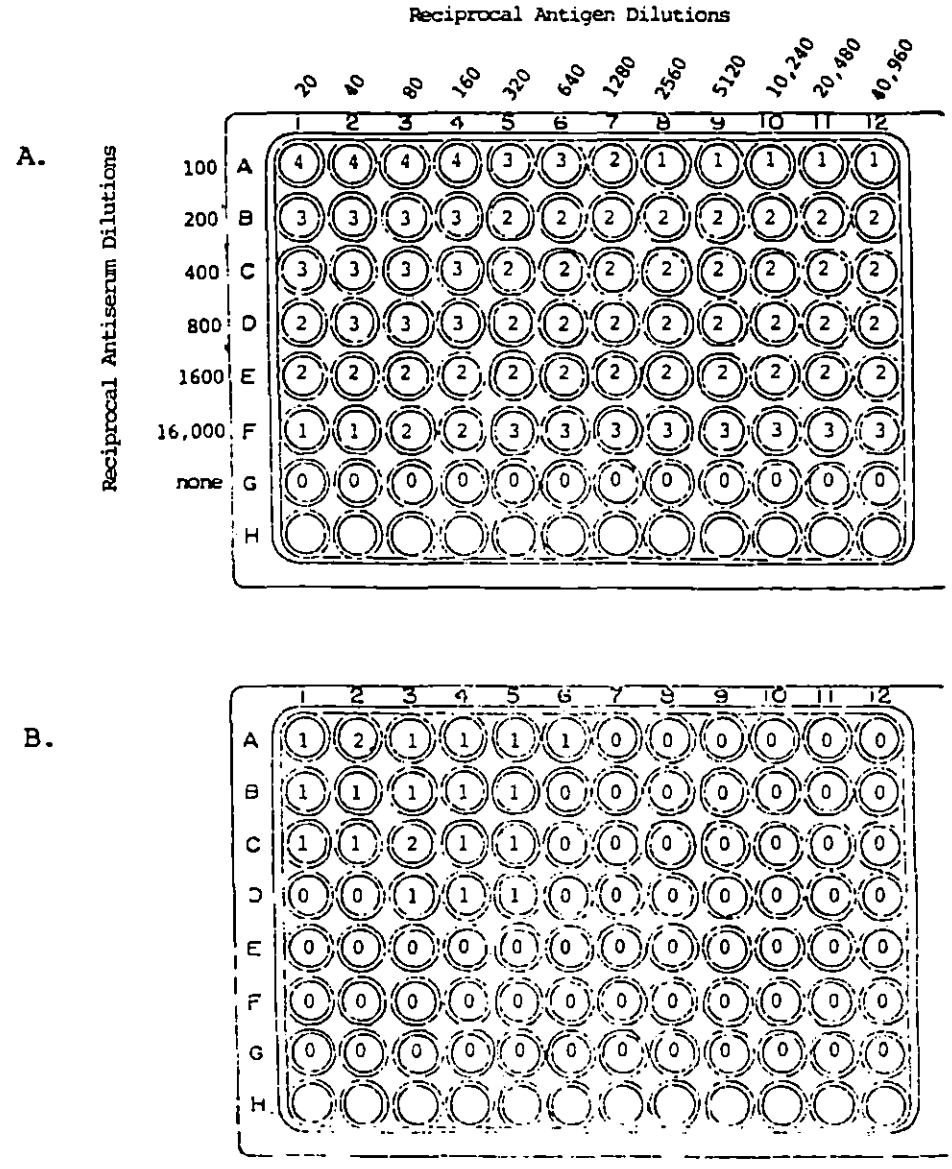


Figure 3. Modified indirect ELISA using S. cholerae-suis O-antigen as coating antigen. Numbers indicate visual color readings ranging from 0 (negative) to 4 (most intense). A. Carbodiimide-activated coating of antigen. B. Passive coating.

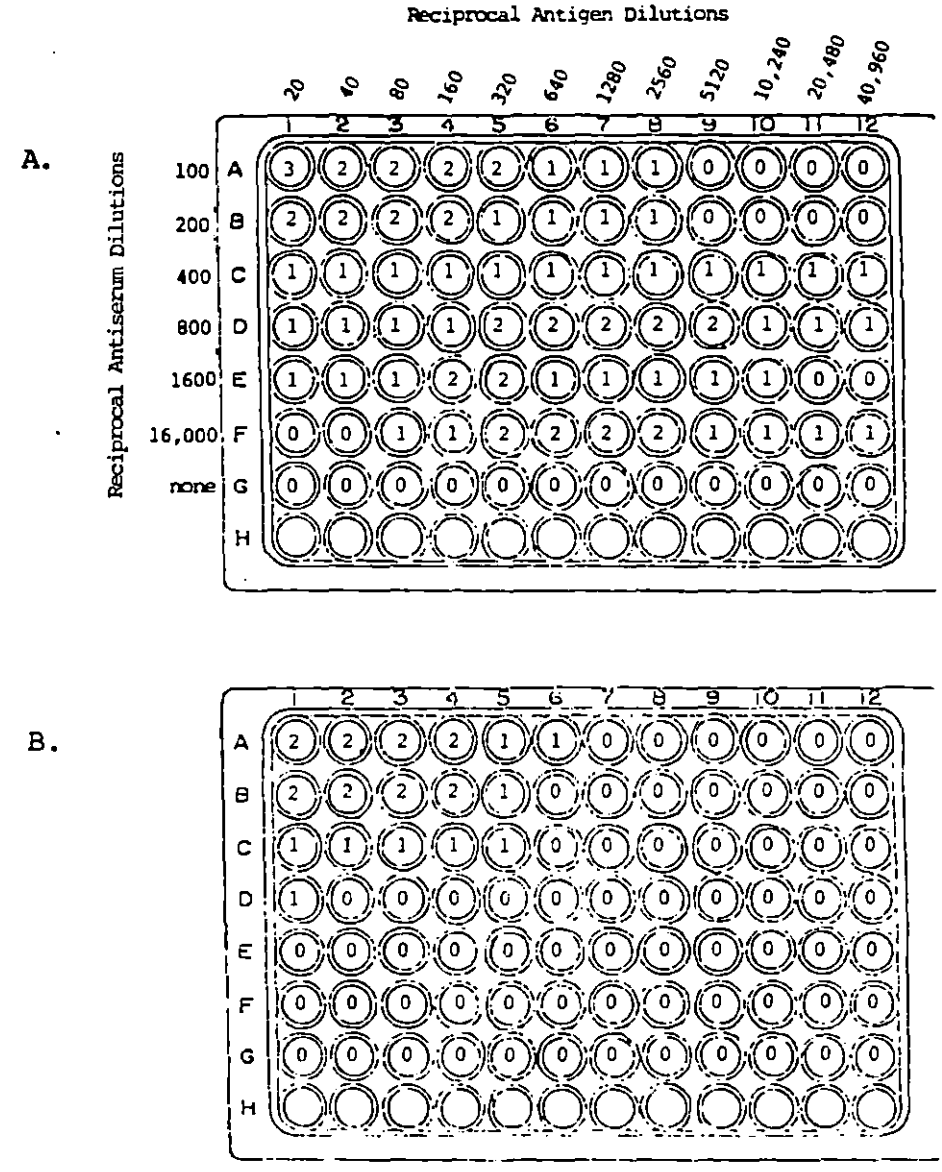


Figure 4. Modified indirect ELISA using S. dublin O-antigen as coating antigen. Numbers indicate visual color readings ranging from 0 (negative) to 4 (most intense). A. Carbodiimide-activated coating of antigen. B. Passive coating.

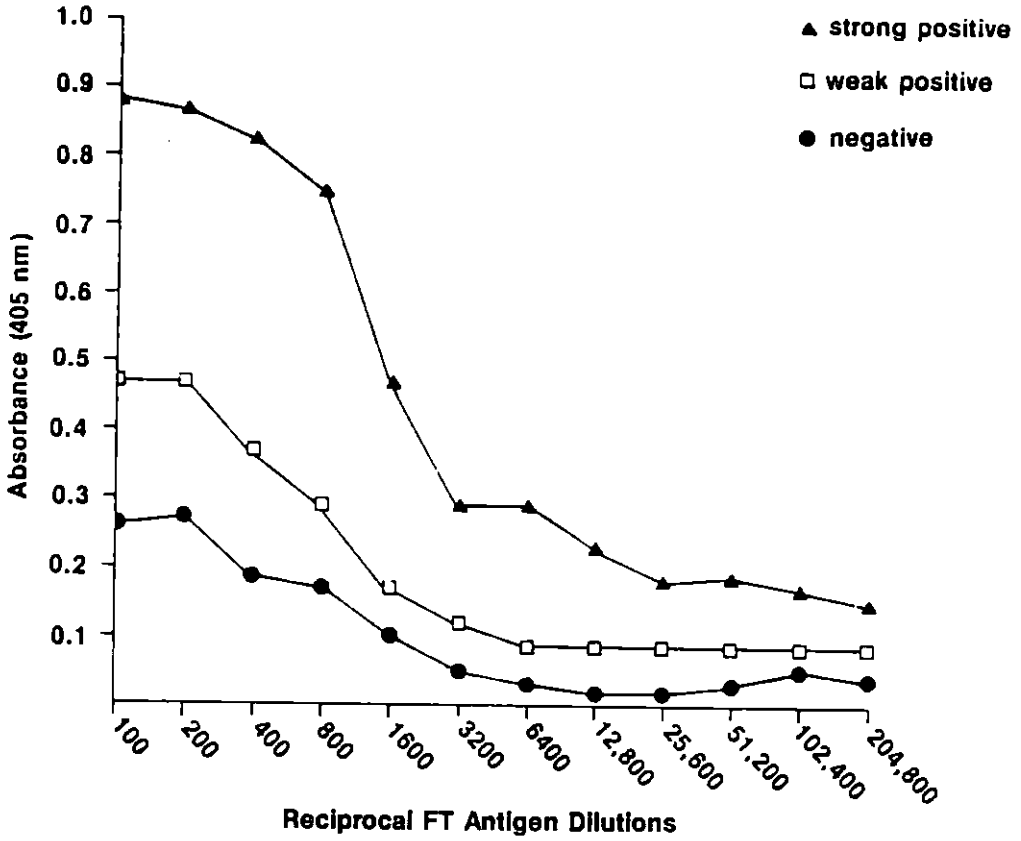


Figure 5. Titration of coating antigen, S. cholerae-suis freeze-thaw (FT) antigen, in the indirect ELISA. Porcine anti-S. cholerae-suis antibody was used at a dilution of 1:100 and rabbit anti-porcine peroxidase at 1:200. Points indicate the mean of duplicate values. The graph indicates the optimum dilution of FT to be 1:500.

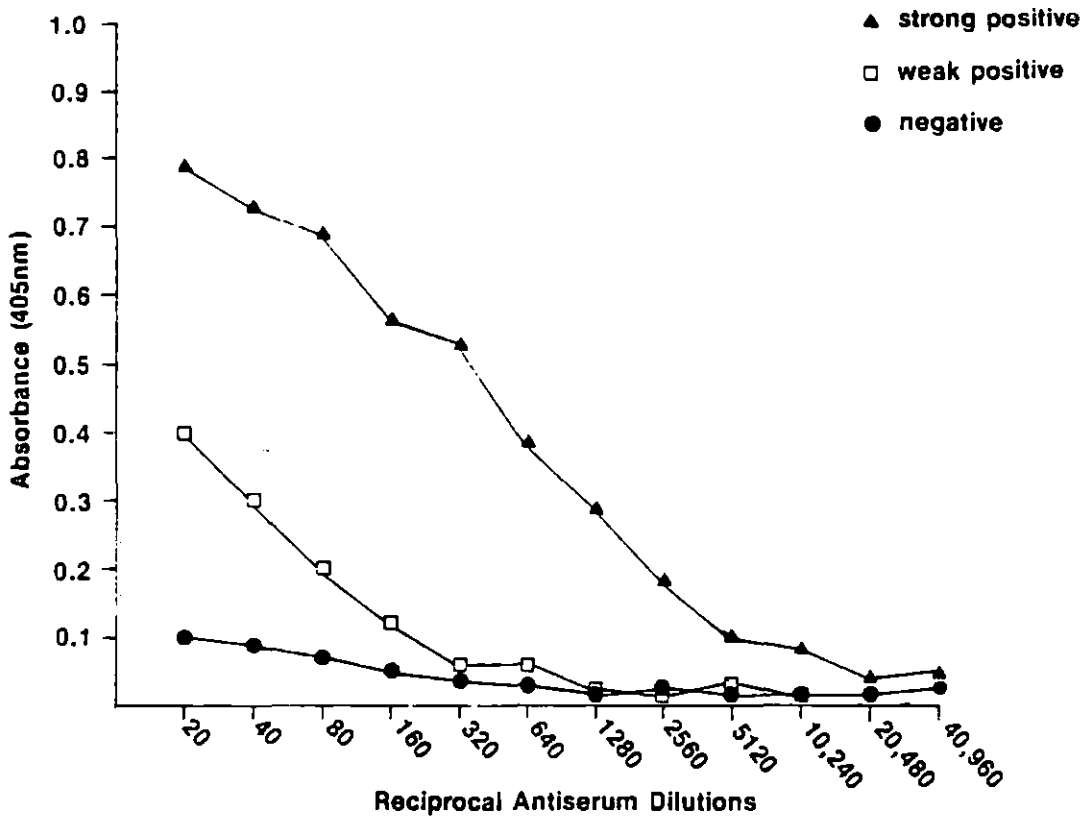


Figure 6. Titration of antiserum, porcine anti-S. cholerae-suis, in the indirect ELISA. FT antigen was coated to the plate at a 1:500 dilution and rabbit anti-porcine peroxidase was diluted 1:200. Points indicate the mean of duplicate values. The graph indicates the optimum antiserum dilution to be 1:80.

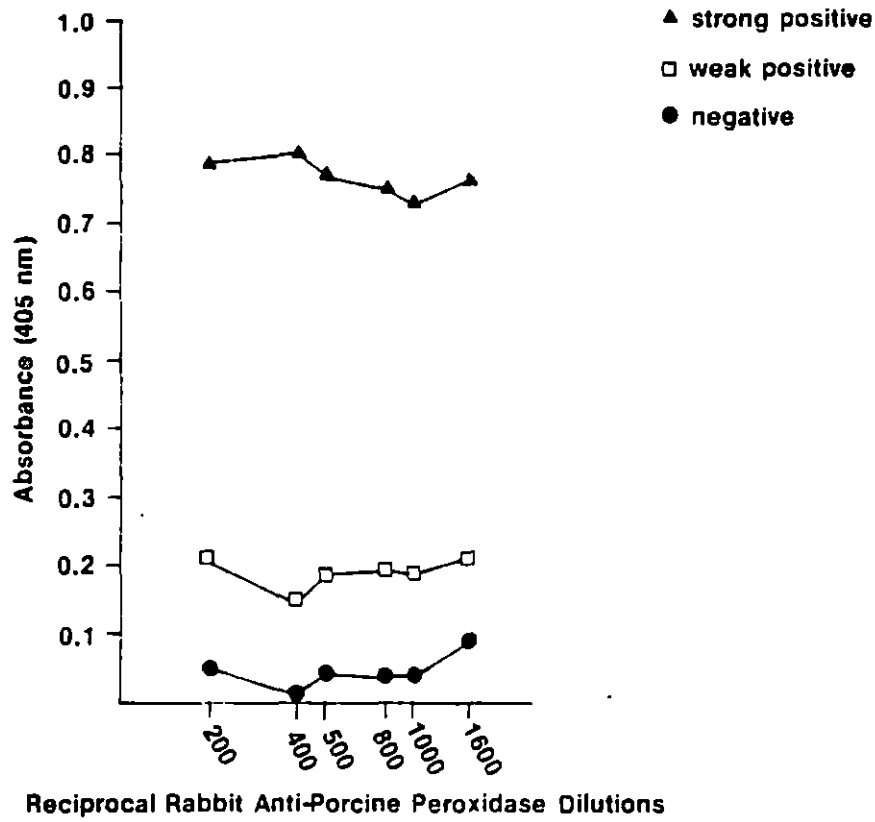


Figure 7. Titration of rabbit anti-porcine peroxidase in the indirect ELISA. FT antigen was coated to the plate at a 1:500 dilution and porcine anti-S. cholerae-suis was diluted 1:80. Points indicate the mean of duplicate values. The graph indicates the optimum antiserum dilution to be 1:400.

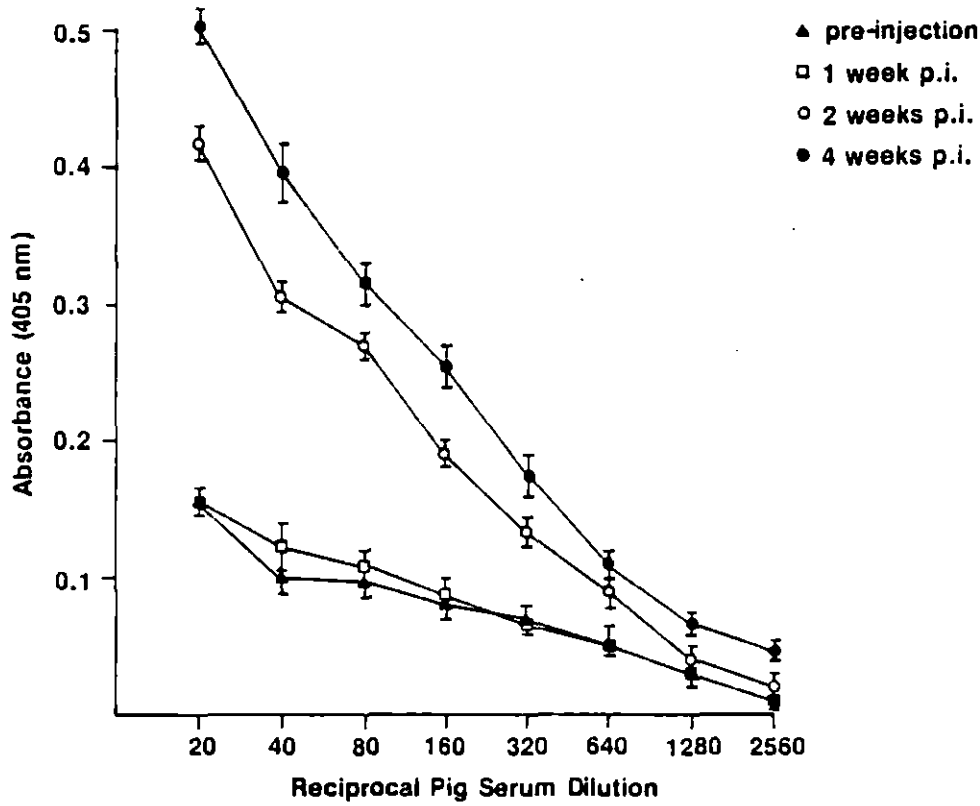


Figure 8. Indirect ELISA results for pigs in Groups 1 and 4, which were injected with S. cholerae-suis only. Points indicate the mean of 10 pigs ± SEM.

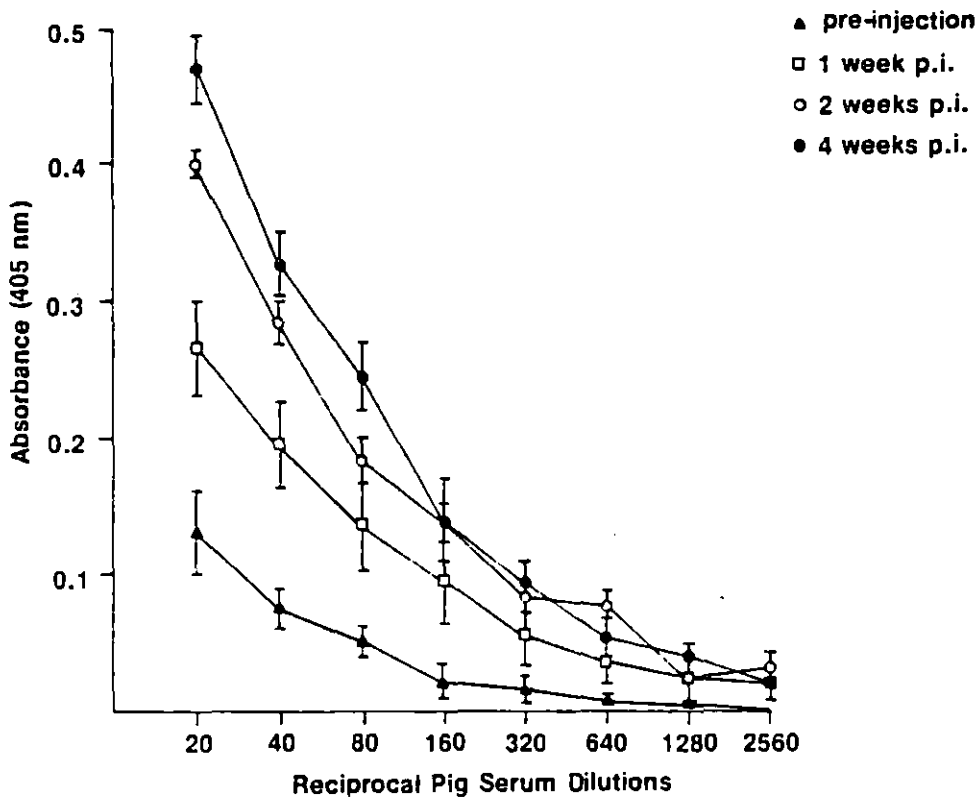


Figure 9. Indirect ELISA results for pigs in Group 2, which were injected with CY on days 0, 2, and 4 and S. cholerae-suis on day 0. Points indicate the mean of 3 pigs ± SEM.

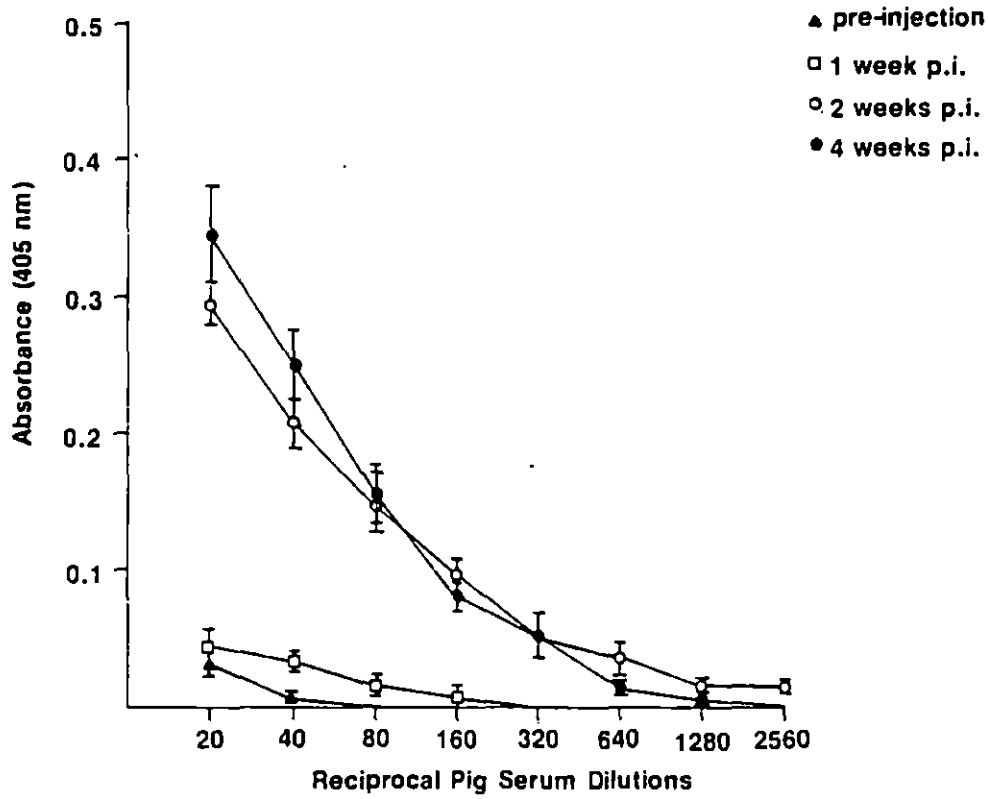


Figure 10. Indirect ELISA results for pigs in Group 3, which were injected with CY on days 0, 2, and 4 and S. cholerae-suis on day 4. Points indicate the mean of 5 pigs ± SEM.

PART II.

DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY TO DETECT *SALMONELLA CHOLERAE-SUIS* VAR. *KUNZENDORF* ANTIGENS IN PORCINE TISSUES

Summary

The solid-phase enzyme-linked immunosorbent assay (ELISA) was investigated for its efficacy in detecting *Salmonella cholerae-suis* var. *kunzendorf* antigens in fresh porcine gut tissues and enrichment broths from tissues and feces. Two types of assays for antigen detection were developed: an inhibition ELISA and a modified double-antibody sandwich ELISA. The inhibition ELISA was not as sensitive as the sandwich ELISA, did not succesfully detect *S. cholerae-suis* antigens in fresh tissue extracts, and therefore was not tested further. The sandwich ELISA detected as little as 60 ng/ml soluble protein antigen and demonstrated a 77% sensitivity and 74% specificity on extracts from fresh gut tissues of pigs experimentally infected with *S. cholerae-suis*. The sandwich ELISA was also tested on enrichment broths inoculated with tissues and feces from infected pigs. Selenite broth was chosen as the enrichment medium because it did not cause high background readings as did tetrathionate broth in the ELISA. By the sandwich ELISA bacteriologically positive enriched fecal samples were not identified but bacteriologically positive

tissue enrichments were detected with 73% sensitivity and 100% specificity. The enzyme immunoassays developed were not adequately sensitive to supplant routine bacterial culture for this organism.

Introduction

Conventional methods of Salmonella cultivation and identification from animal tissues require 48-72 hours. In cases when a herd outbreak is suspected or animals are tested for transport, a more rapid diagnosis would be desirable since considerable economic losses can result.[181] The enzyme-linked immunosorbent assay (ELISA) was studied in this project for efficacy in identifying S. cholerae-suis var. kunzendorf antigens in porcine tissues. ELISA was chosen over other immunoassays for antigen detection, such as counterimmunoelectrophoresis, immunofluorescence, and radioimmunoassay because of its potential for high sensitivity, objective interpretation, and need for simple equipment.

Several sensitive ELISAs have been developed for the detection of bacterial antigens in urine, sputum, cerebrospinal fluid, serum, and feces.[10,50,55,60,77,178] Identification of Salmonella antigens by ELISA has been limited to food microbiology.[103,118,134,147] Since S. cholerae-suis is usually harbored in the feces or intestinal tissues of a carrier pig,[181] we applied the ELISA to the detection of antigens from this organism directly in gut tissues and in enrichment broths

from feces and various body tissues. The use of enrichment is important to increase the number of Salmonellae and application of an ELISA can shorten the identification period by as much as 24 hours. This study involved the selection of a suitable enrichment broth, the development of an inhibition ELISA and a double-antibody sandwich ELISA, the determination of the sensitivity of these tests, and their application to the detection of S. cholerae-suis antigens from porcine tissues and enrichment broths.

Materials and Methods

Development of an inhibition ELISA for antigen detection

Dynatech Immulon I flat-bottomed polystyrene microtiter plates were coated with 100 microliters per well of a 1:500 dilution of S. cholerae-suis var. kunzendorf freeze-thaw antigen (FT, see Part I) in sodium carbonate, pH 9.6. Fifty microliters of a 0.2 mg/ml carbodiimide solution in sodium carbonate were added per well according to a previously described procedure[2] and the dilution of FT (1:500) was already determined optimum (see Part I). After overnight coating, plates were washed 3 times in PBS, incubated 30 min in 0.1M NH_4Cl, and again washed 3 times in PBS before the addition of subsequent reagents.

Primary antiserum, porcine anti-S. cholerae-suis, was obtained from a gilt hyperimmunized with live organisms and O-antigen on three

occasions and had an O-agglutination titer of 1:640 and an indirect ELISA titer of 1:2560. An aliquot of this serum was precipitated with ammonium sulfate and subjected to affinity purification on an Affi-Gel 10 column (Bio-Rad) activated with FT antigen. The final antiserum fraction had an indirect ELISA titer of 1:2560. Various dilutions of this fraction with and without S. cholerae-suis O-antigen were incubated together, in a checkerboard manner, 50 microliters each per well, on a separate microplate for 30 min at room temperature on a shaker before adding to the plate coated with FT. The antibody-O-antigen mixtures were then transferred well-by-well to the coated plate and incubated for 30 min on a shaker as before. The plate was emptied, washed 8 times in PBS-Tween, and the conjugate was added. The conjugate, rabbit anti-pig peroxidase (Cappel) was added in 100 microliter quantities per well at a 1:400 dilution, previously determined to be optimum (see Part one). Incubation was 30 min as before, followed by 8 washes and the addition of substrate, ABTS (Sigma) and hydrogen peroxide in 0.05M citric acid for 60 min.

Using the optimum dilution of antiserum, the limiting sensitivity of the inhibition ELISA to detect O-antigen and whole bacterial cells was determined (see Results).

Development of a modified double-antibody sandwich ELISA for antigen detection

Anti-S. cholerae-suis antibody was produced in chickens by repeated intravenous injections of a heat-killed pool of 4 strains of

the organism. Chickens were bled before injection and 3 weeks after the first injection. Chicken was chosen as the coating antibody because cross-reactions were observed between porcine and rabbit antibody in the sandwich ELISA. Control background levels were sufficiently low between chicken and rabbit antisera in this assay. All antisera were precipitated in ammonium sulfate for use in the ELISA.

The optimal dilution of chicken antibody coating the plate was determined by titration (see Results). Twofold dilutions of chicken antibody were added to the plate in 100 microliter quantities per well in sodium carbonate, pH 9.6. Fifty microliters of a carbodiimide solution were then added per well as previously described and the plate incubated overnight at 4C. The next day plates were emptied, washed 3 times in PBS, and incubated in 0.1M ammonium chloride for 30 min. This was followed by 3 washes in PBS and the addition of 100 microliters per well of 1:500 FT for 30 min. Plates were washed 8 times in PBS-Tween followed by the addition of 100 microliters/well of 1:100 rabbit anti-S. cholerae-suis for 30 min. After 8 more washes in PBS-Tween, the conjugate, goat anti-rabbit peroxidase, was added in 100 microliter quantities per well at a 1:200 dilution for 30 min. Finally, the plates were washed 8 times and the substrate solution, ABTS and hydrogen peroxide, was added for 30 min. By utilizing the coating antibody at optimal dilution (see Results), the rabbit anti-S. cholerae-suis was titrated in a similar manner. The optimal dilution of this antiserum was also determined (see Results). The limiting

sensitivity of this ELISA to detect FT antigen and whole S. cholerae-suis cells was determined (see Results).

Collection and preparation of porcine tissues

Gut tissues from 12 pigs sacrificed serially after infection were quick frozen in liquid nitrogen and stored at -70C. Some of these pigs were vaccinated with 4.3×10^8 S. cholerae-suis var. kunzendorf intramuscularly and all were challenged with 1.5×10^9 organisms intratracheally 14 days later.[69] Tissues collected were: jejunum, ileocecal junction, cecum, colon, and gall bladder. A portion of each tissue was placed in tetrathionate broth (BBL) for culture before freezing. These enrichment broths were subcultured after 18 hours onto brilliant green agar (BG, Difco Laboratories, Detroit, MI) and suspect lactose-negative colonies were inoculated on Kligler's iron agar and urea agar slants (Difco). Growth characteristic of S. cholerae-suis was identified serologically using plate agglutination and commercial O-typing sera (Fisher Scientific Co., Pittsburgh, PA).

To prepare samples for the ELISA, tissue segments were thawed and gently ground in 1 ml sterile saline to free the gut contents and adhering mucus. Samples were placed in 1 dram glass vials, heat-treated at 60C for 1 hour, and stored at -70C. Immediately before testing in the ELISA, samples were thawed and centrifuged at 1000 rpm for 10 min to remove large particulate material.

Enrichment of tissue samples

Porcine tissues were collected from the Iowa State Veterinary Diagnostic Latoratory suspected of S. cholerae-suis infection. Approximately 1 gram portions were chopped and placed in 10 ml of selenite broth (Difco). After overnight incubation at 37C, broths were subcultured onto BG agar and colonies suspected to be S. cholerae-suis were isolated and identified by the method previously described. Selenite broths were stored at -70C. Before testing in the ELISA, the broths were heat-treated at 60C for 2 hours followed by centrifugation at 1000 rpm for 10 min.

Rectal swabs were collected from 48 pigs experimentally infected with 2.2×10^9 S. cholerae-suis var. kunzendorf intratracheally. Duplicate swabs from each pig were collected on 3 occasions at 2-day intervals after infection and were placed in one tube each of tetrathionate and selenite broths for overnight enrichment at 37C. Identification of S. cholerae-suis was performed as described previously. Selenite broths were prepared for testing in the ELISA as above.

Testing of tissues and enrichment broths by ELISA

Samples were added in 100 microliter per well quantities in quadruplicate according to the previously described inhibition and sandwich ELISA protocols. To account for the varying composition of each sample, each was tested against two wells coated with immune chicken antiserum and two wells coated with non-immune (negative) chicken serum. Substrate was incubated 30-60 min on the plates,

depending on the time of appearance of the best contrast between absorbance values with positive and negative sera.

Results

Bacterial culture

Table 1 presents the culture results for rectal swabs enriched in tetrathionate versus selenite. Selenite broth was chosen as the enrichment medium of choice because very high backgrounds were observed in the sandwich ELISA when tetrathionate was used (Fig. 8). Table 2 summarizes the culture results for the gut tissues tested in the inhibition and sandwich ELISA. Table 3 displays the selenite broths positive for *S. cholerae-suis* from tissues and rectal swabs.

Inhibition ELISA

The titration of porcine anti-*S. cholerae-suis* antibody is displayed in Fig. 1. The optimal dilution of antibody was 1:100 when incubated with 1:1000 O-antigen, the dilution of antigen exhibiting the best inhibition of reaction. Using the optimum dilution of antiserum, the limiting sensitivity of the inhibition ELISA to detect O-antigen and whole bacterial cells was determined. Significant inhibition (at least twofold) was observed with a 1:4000 dilution of O-antigen or 10^9 cells/ml (Fig. 2-3).

The inhibition ELISA was used to test the gut tissues from 4 of

the 12 pigs. Results are summarized in Table 4 and are expressed as a P/N value (positive/negative), the ratio of absorbance values for wells coated with positive chicken anti-S. cholerae-suis antibody to negative chicken antibody, respectively. The P/N value for 1:1000 S. cholerae-suis O-antigen (positive control) was 6.0. Since poor correlation was observed between ELISA and culture, the inhibition assay was not tested further.

Modified Double-antibody Sandwich ELISA

Titration results for coating antibody, chicken anti-S. cholerae-suis, and second antibody, rabbit anti-S. cholerae-suis, are displayed in Fig. 4-5. Optimal dilutions were interpreted as in Part I (pp. 46-47). Both sera were utilized at a 1:5000 dilution.

The limiting sensitivity of this ELISA to detect FT antigen and whole cells was 60 ng/ml (1:32,000 of a 1.92 mg/ml solution) and 10^7 cells/ml, respectively (Fig. 6-7). This was determined by selecting the last point on the graph with an absorbance value above 0.1 while the negative serum control remained below this value.

Results from the testing of gut tissues are summarized in Table 5. By setting the P/N value as 1.5 or greater, the best correlation between ELISA and culture was obtained. Enrichment results are summarized in Tables 6 and 7.

Sensitivity and specificity

The overall sensitivity of the modified double-antibody sandwich

ELISA to detect S. cholerae-suis antigens in direct tissue samples was calculated by the following formulas:[21]

$$\text{Sensitivity} = \frac{\text{no. true positives}}{\text{no. true pos. + false neg.}} \times 100 = 77\%$$

$$\text{Specificity} = \frac{\text{no. true negatives}}{\text{no true neg. + false pos.}} \times 100 = 74\%$$

True positives and negatives refered to culture results. This ELISA was unable to detect positive fecal enrichments, with a sensitivity of only 10%. The sensitivity and specificity in detecting positive tissue enrichments as calculated by the above formulas were 73% and 100%, respectively.

Discussion

The enzyme immunoassays developed in this study were insufficiently sensitive to supplant routine bacterial culture for S. cholerae-suis. Since the sandwich ELISA detected as little as 60 ng/ml soluble protein antigen, results from the testing of tissues and enrichments indicate that many samples contain less soluble antigen or those antigens are masked by other components in the sample. The amount of soluble antigen released into the tissue or medium is important since the ELISA was inefficient in detecting whole cells (Fig. 3 and 7). It is known that in vitro cultures of Salmonella release a great deal of antigenic material into the medium as the culture passes into the stationary phase.[37] Multiplying organisms in

the actively infected host reportedly release antigens steadily and the destruction of organisms by the cell-mediated immune response results in the release of large amounts of LPS and nucleoprotein into the tissues.[36] The inability of the ELISA to detect these antigens could be explained by a variety of factors: the association or uptake of the antigens by macrophages and neutrophils,[36] antigen reacting with antibody in the bile or gut to form immune complexes,[22,137,138] or the nature of the antibody idiotypes used in the ELISA to detect antigen. In retrospect, antibody produced against a whole-cell bacterin such as that used in this study probably cannot react with all antigens resulting from bacterial autolysis.

Purity of the antigen in question also contributes to sensitivity. More sensitive ELISAs have been developed for the detection of Escherichia coli heat-labile enterotoxin,[186] Haemophilus influenzae type b antigen (polyribose phosphate),[178] Streptococcus pneumoniae type 3 antigen,[50,77] and staphylococcal enterotoxins.[157] Sensitivities ranged from 0.1 pg/ml to 3.0 ng/ml. The most sensitive assay was for E. coli heat-labile enterotoxin[186], but interestingly, required approximately 2 days for completion. Variables influencing sensitivity also include the type of ELISA and the nature of the specimen tested. Feces and complex body fluids have resulted in lower sensitivities, such as 1.0 μg/ml for entertoxigenic E. coli colonization factor antigen I[60] and E. coli K99 antigen at a level of 10^7 cells/ml.[55] Highly sensitive assays for antigen have been developed for cerebrospinal fluid[50,77,178] and urine.[10,50] The

impurity of the Salmonella antigen and the clinical specimens used in our study probably contributed to the inability to create a more sensitive assay.

More sensitive enzyme immunoassays have also been developed for the detection of Salmonella in foods.[103,118,134,147] Sensitivities ranged from 10^3-10^6 cells/ml but the assays were not tested for reactivity with soluble antigen. The most sensitive test utilized a monoclonal antibody bound to alkaline phosphatase in a competitive solid-phase ELISA.[147] This assay involved alkaline phosphatase-labeled antibody which detected common Salmonella flagellar antigens in raw milk or culture media at a concentration of 10^3 cell/ml. Antigens competed with polymerized flagellin bound to polyvinyl microplates for the binding of the enzyme-labeled antibody. The greater sensitivity of this assay is probably due to the specificity and purity of the monoclonal antibody preparation. The monoclonal antibody is also advantageous in that it can be directly conjugated to the enzyme without a great loss of specificity, which is a problem with conventional antibody preparations. The result was a procedure with fewer steps, decreased background readings, and longer incubations which increased sensitivity, while the test still remained within reasonable working time.

Enrichment is an important procedure for Salmonella detection in both food and veterinary microbiology. Choice of enrichment medium for animal Salmonellae is apparently a matter of preference, with tetrathionate broth perhaps most widely employed. Selenite is

preferred by some laboratories (Dr. B. O. Blackburn, personal communication) because it reportedly inhibits Proteus spp. to a greater extent and allows the growth of some Salmonella serotypes better than tetrathionate.[46] However, both media have been reported to inhibit the growth of S. cholerae-suis to a certain extent.[52] Results from this study indicate little difference between the ability of tetrathionate and selenite to support the growth of selected strains of S. cholerae-suis var. kunzendorf. Table 1 shows little correlation between fecal samples diagnosed as positive using selenite and tetrathionate. This supports the inhibition of growth by both media as well as the importance of colony selection from the plate, in this study using brilliant green agar. The failure of this ELISA to detect positive fecal enrichments may reflect in part the inhibition of bacterial growth by selenite so that enough antigen is not released for detection.

The most successful assay developed was the modified double-antibody sandwich ELISA to detect S. cholerae-suis in direct tissue samples and enrichment broths from tissues. This could be most useful in screening carcasses in slaughterhouses or in diagnostic laboratories, where a large number of animals are tested daily for Salmonella infection. A polyvalent assay would be highly suitable especially in the abattoir.[109,147] Modification of the many variables in this ELISA may result in a more sensitive test, but the major challenge remains the development of a test adequately simple and rapid to be of use for the diagnostician.

Table 1: Comparison of tetrathionate and selenite broths for recovery of S. cholerae-suis var. kunzendorf from rectal swabs of pigs experimentally infected. Underlined numbers indicate positive samples detected by both tetrathionate and selenite. The numbers in parentheses are percentages of total possible positive cultures detected by each medium

Collection, days p.i.	Pig Nos. positive by	
	Selenite	Tetrathionate
2	13,15,20,21 (100%)	none (0%)
4	6,9,14,17,18, 19,20,23,26,27, 31,38,47 (68%)	4,11,13,14,17, 18,19,24,35,43 47 (58%)
8	17,19,36,42 (44%)	5,19,27,35,39, 43 (67%)

Table 2: Bacterial culture results for S. cholerae-suis var. kunzendorf in intestinal samples

Pig No.	jejunum	ileocecal jctn.	cecum	colon	bile
93	+	+	+	+	-
94	+	+	-	-	+
97	-	+	-	-	-
99	+	+	+	+	-
100	-	-	-	-	-
420	+	+	+	-	-
422	-	-	-	-	-
425	-	+	-	-	-
429	+	+	+	+	-
431	+	-	-	-	+
432	-	+	+	-	-
532	-	-	-	-	-

Table 3: Recovery of S. cholerae-suis var. kunzendorf in selenite enrichment broth by bacterial culture

Culture positive pig numbers obtained by rectal swabs
Day 2: 13, 15, 20, 21
Day 4: 6, 9, 14, 17, 18, 19, 20, 23, 26, 27, 31, 38, 47
Day 8: 17, 19, 36, 42

Tissues

Sample No.	Tissue	Culture
1	lung	+
2	liver	+
3	spleen	-
4	jejunum	-
5	ileum	-
6	cecum	-
7	colon	+
8	mesenteric l.n.	-
9	spleen	+
10	lung	+
11	bronchial l.n.	+
12	lung	+
13	liver	+
14	spleen	+
15	colon	+
16	small intestine	+
17	intestine	-
18	small intestine	-
19	spleen	-
20	spleen	-
21	small intestine	-
22	liver	-

Table 4: Inhibition ELISA results from fresh porcine gut tissues (expressed as a P/N value). * indicates tissues positive for S. cholerae-suis by culture. The P/N value is a ratio of the absorbance values obtained from wells coated with positive S. cholerae-suis serum to wells coated with negative serum. If a positive reaction is considered as a P/N value of 1.5 or greater, the sensitivity of the inhibition ELISA when compared to culture was 71% (as calculated in Results). Specificity was 67%

Pig No.	jejunum	ileocecal jctn.	cecum	colon	bile
93	1.0*	2.0*	1.5*	1.4*	1.0
99	1.1*	1.5*	3.4*	2.3*	1.5
100	1.3	2.8	2.0	1.6	1.3
429	1.7*	1.2*	1.7*	1.3*	1.1

Table 5: Sandwich ELISA results from fresh porcine gut tissues (expressed as a P/N value). * indicates culture positive tissues. The P/N value is a ratio of the absorbance values obtained from wells coated with positive S. cholerae-suis serum to wells coated with negative serum. Sensitivity of the sandwich ELISA to detect S. cholerae-suis antigens in fresh tissue was 77% (determined as in Table 4). Specificity was 74%

Pig No.	jejunum	ileocecal jctn.	cecum	colon	bile
93	1.7*	3.0*	3.4*	1.5*	0.7
94	2.0*	1.6*	2.3	1.4	0.0*
97	4.6	1.3*	2.9	1.7	0.5
99	0.9*	1.0*	2.9*	2.4*	0.15
100	1.0	1.3	1.1	1.3	1.2
420	37.0*	1.9*	1.6*	1.4	1.1
422	1.1	2.7	1.4	0.8	0.03
425	4.3	0.7*	2.1	1.5	0.0
429	3.4*	0.7*	5.9*	1.6*	0.3
431	1.1*	4.0	0.8	1.6	0.0
432	2.5	2.1*	2.3*	3.5	0.15
532	1.3	1.8	1.4	1.3	0.0

Table 6: Sandwich ELISA results from fecal enrichments (P/N values). * indicates enrichments positive for S. cholerae-suis by culture. The P/N value is a ratio of the absorbance values obtained from wells coated with positive S. cholerae-suis serum to wells coated with negative serum. Sensitivity of the sandwich ELISA to detect positive fecal enrichments was 10% (determined as in Table 4). Specificity was 83%

Pig No.	Day 2 p.i.	Day 4 p.i.	Day 8 p.i.
1	1.8	0.5	1.1
2	1.5	ND[a]	ND
3	1.3	0.4	1.3
4	1.0	0.4	1.0
5	1.9	0.0	0.9
6	1.6	0.0*	1.4
7	2.0	0.0	0.3
8	1.1	0.0	0.5
9	1.0	0.1*	ND
10	1.0	0.3	ND
11	2.1	0.4	ND
12	1.5	0.4	ND
13	1.7*	0.3	ND
14	1.1	0.0*	0.2
15	1.5*	0.0	ND
16	1.4	0.4	0.3
17	1.8	0.1*	0.2*
18	0.8	0.2*	0.3
19	1.5	0.0*	0.3*
20	1.6*	0.2*	0.3
21	1.2*	0.5	0.1
22	ND	ND	ND
23	1.0	0.5*	0.2
24	1.2	0.7	0.4
25	1.5	0.2	0.1
26	1.2	0.2*	0.1
27	1.7	0.3*	0.5
28	1.6	1.0	0.0
29	1.3	0.2	0.0
30	0.9	0.0	0.1
31	1.5	0.0*	0.2
32	3.5	0.2	0.0
33	0.9	0.6	0.0
34	1.4	0.1	0.0
35	1.0	0.1	0.1
36	2.0	0.3	0.1*
37	1.4	0.2	0.1

[a]ND=not determined, due to death of the animal.

Table 6:(continued)

Pig No.	Day 2 p.i.	Day 4 p.i.	Day 8 p.i.
38	1.9	0.7*	0.1
39	1.9	0.3	0.0
40	2.0	0.3	0.0
41	1.1	0.7	0.0
42	1.4	0.1	0.1*
43	1.8	0.4	0.2
44	1.6	0.2	0.1
45	0.9	0.6	0.0
46	2.2	0.2	0.1
47	2.3	0.6*	0.2
48	1.8	0.4	0.9

Table 7: Sandwich ELISA results from tissue enrichments. * indicates enrichments positive for S. cholerae-suis by culture. The P/N value is a ratio of the absorbance values obtained from wells coated with positive S. cholerae-suis serum to wells coated with negative serum. Sensitivity of the sandwich ELISA to detect positive tissue enrichments was 73% and specificity was 100%

Sample	P/N value	Tissue	P/N value
1-lung	0.6*	12-lung	2.2*
2-liver	0.8*	13-liver	2.7*
3-spleen	0.7	14-spleen	1.6*
4-jejunum	0.9	15-colon	2.2*
5-ileum	0.8	16-small int.	1.6*
6-cecum	0.7	17-intestine	0.6
7-colon	0.9*	18-small int.	0.8
8-mes. l.n.	1.0	19-spleen	0.7
9-spleen	1.7*	20-spleen	0.7
10-lung	2.1*	21-small int.	0.6
11-br. l.n.	2.3*	22-liver	0.6

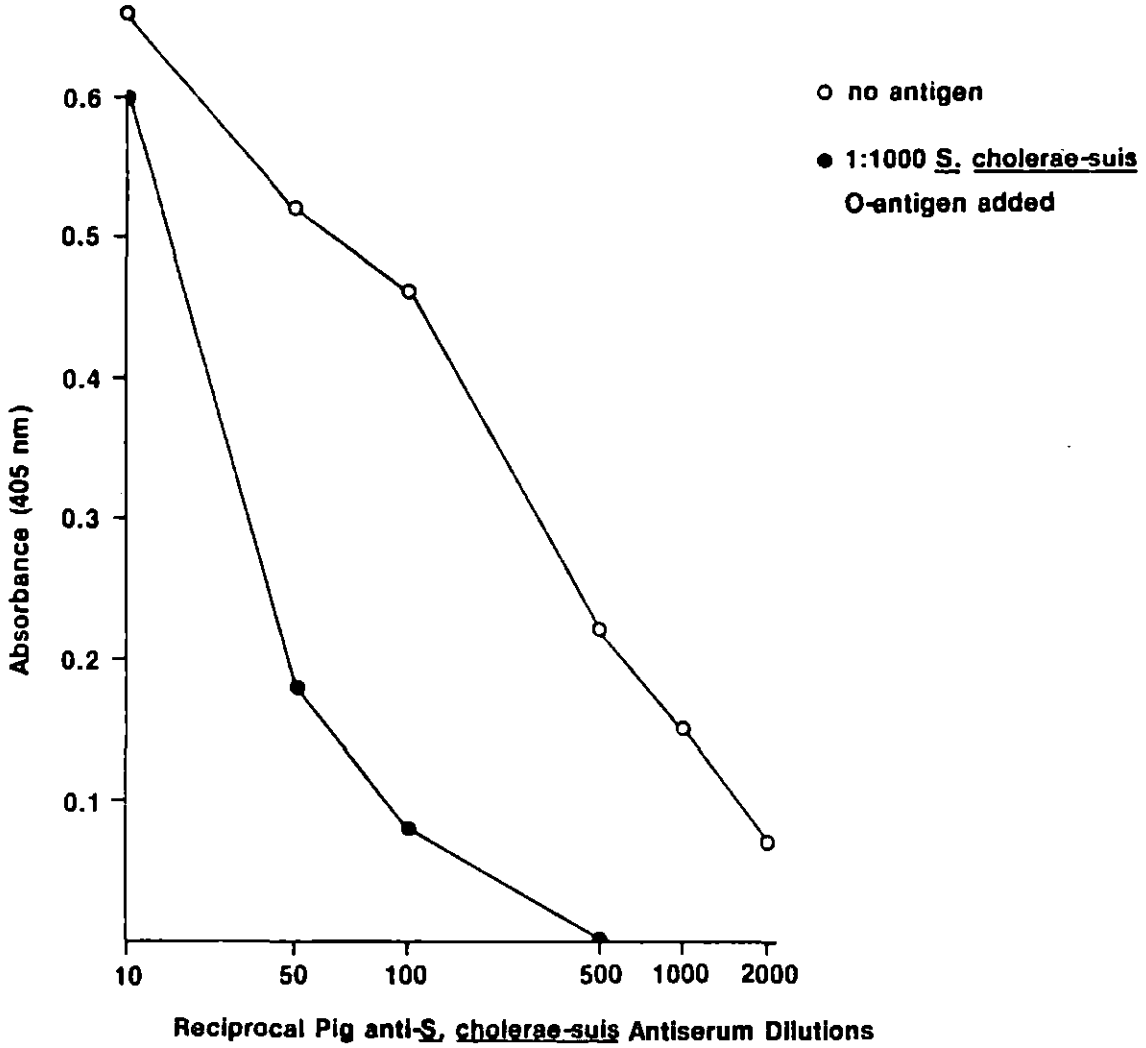


Figure 1. Inhibition ELISA: titration of pig anti-S. cholerae-suis antibody. FT antigen was coated to the plate at a 1:500 dilution, varying dilutions of anti-S. cholerae-suis antibody and O-antigen were incubated together before adding to the coated plate. The 1:1000 dilution of O-antigen is displayed on the graph because it exhibited inhibition of antibody reaction. Rabbit anti-porcine peroxidase was diluted 1:400. Points represent means of duplicate values. The graph shows the optimum antiserum dilution displaying the highest degree of inhibition to be 1:100.

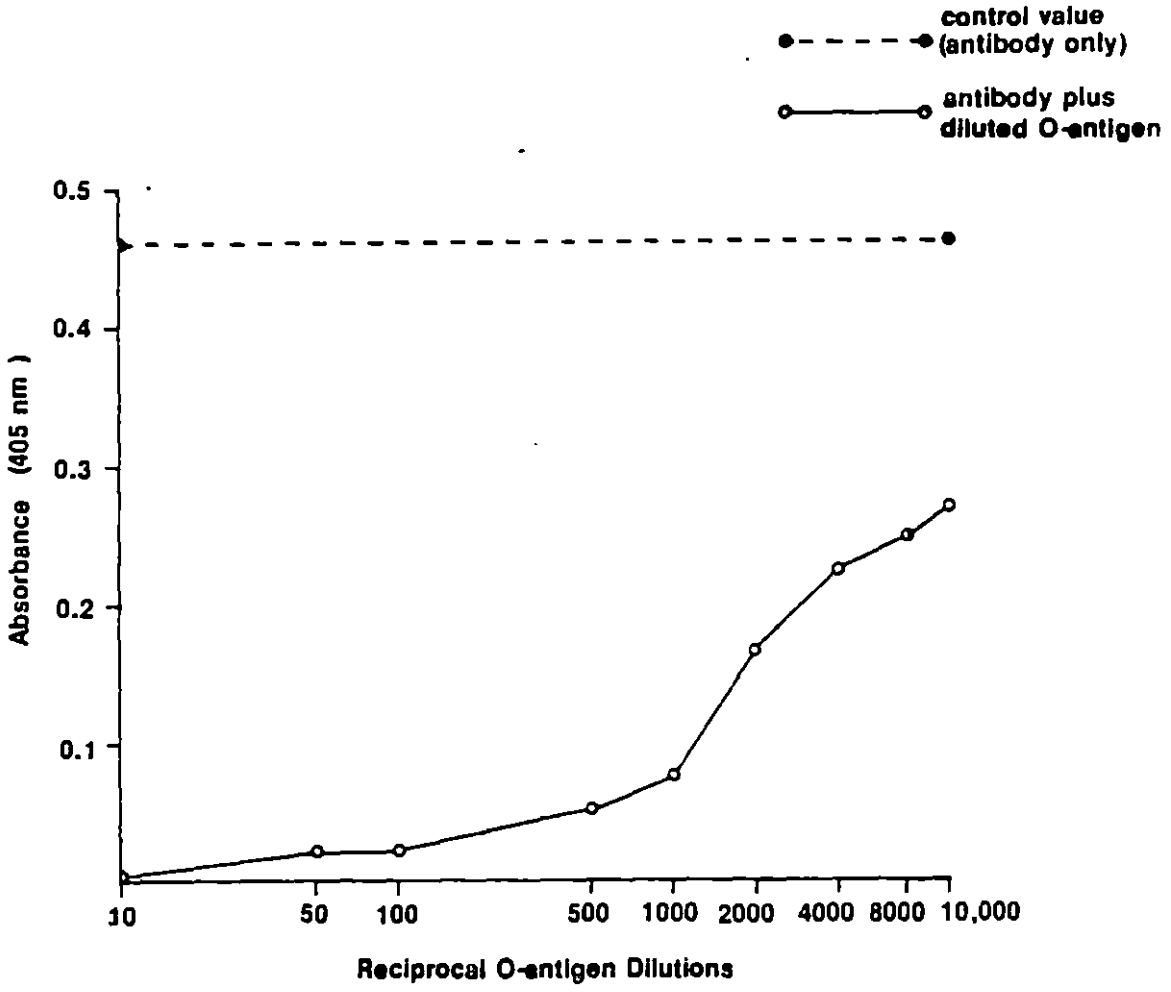


Figure 2. Inhibition ELISA: limiting sensitivity to detect S. cholerae-suis O-antigen. FT antigen was coated to the plate at a 1:500 dilution, pig anti-S. cholerae-suis was diluted 1:100 before adding to varying O-antigen dilutions, and rabbit anti-porcine peroxidase was diluted 1:400. Points represent means of duplicate values. The highest dilution of O-antigen inhibiting the reaction by 2X was 1:4000.

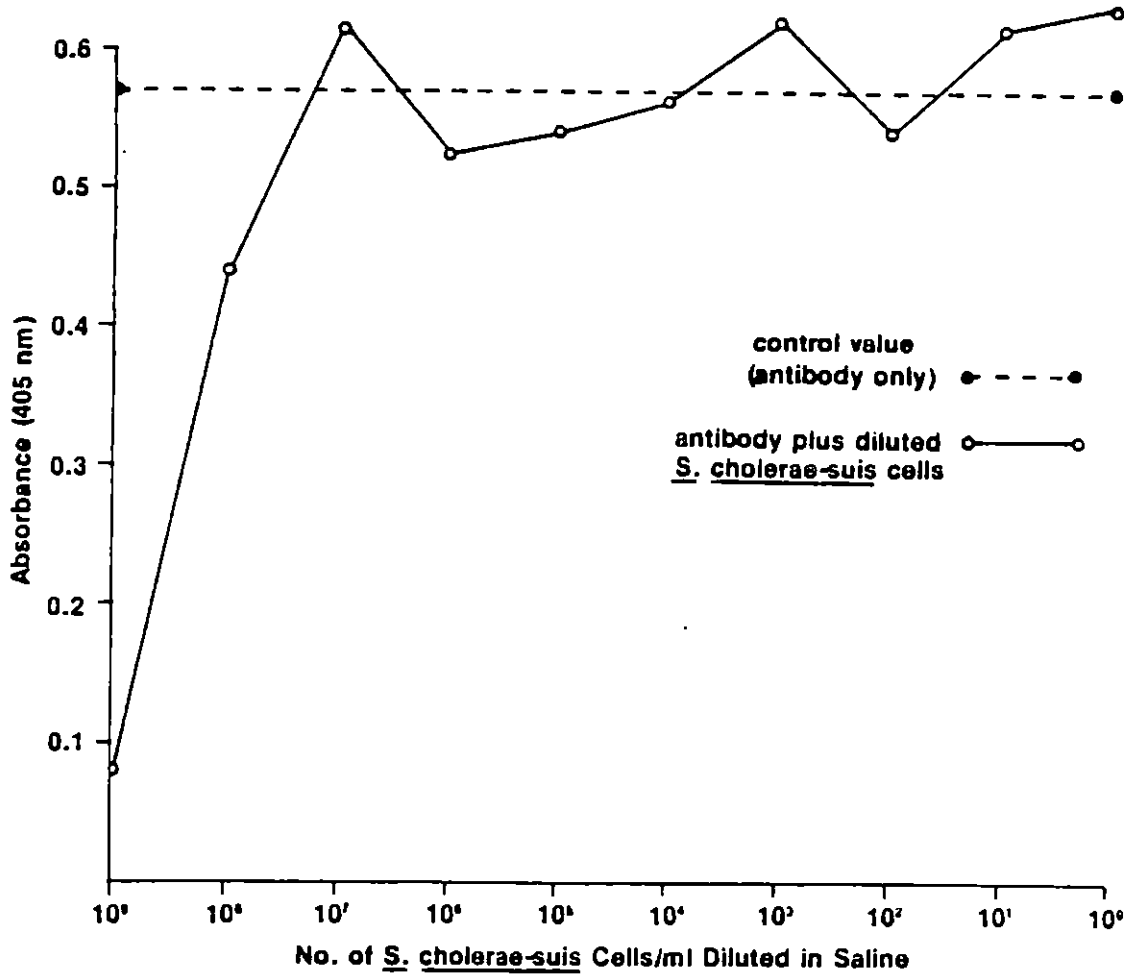


Figure 3. Inhibition ELISA: limiting sensitivity to detect whole S. cholerae-suis cells. FT antigen was coated to the plate at a 1:500 dilution, pig anti-S. cholerae-suis was diluted 1:100 before adding to varying dilutions of S. cholerae-suis in saline, and rabbit anti-porcine peroxidase was diluted 1:400. Points represent the means of duplicate values. The lowest concentration of cells/ml inhibiting the reaction by 2X was 10^9.

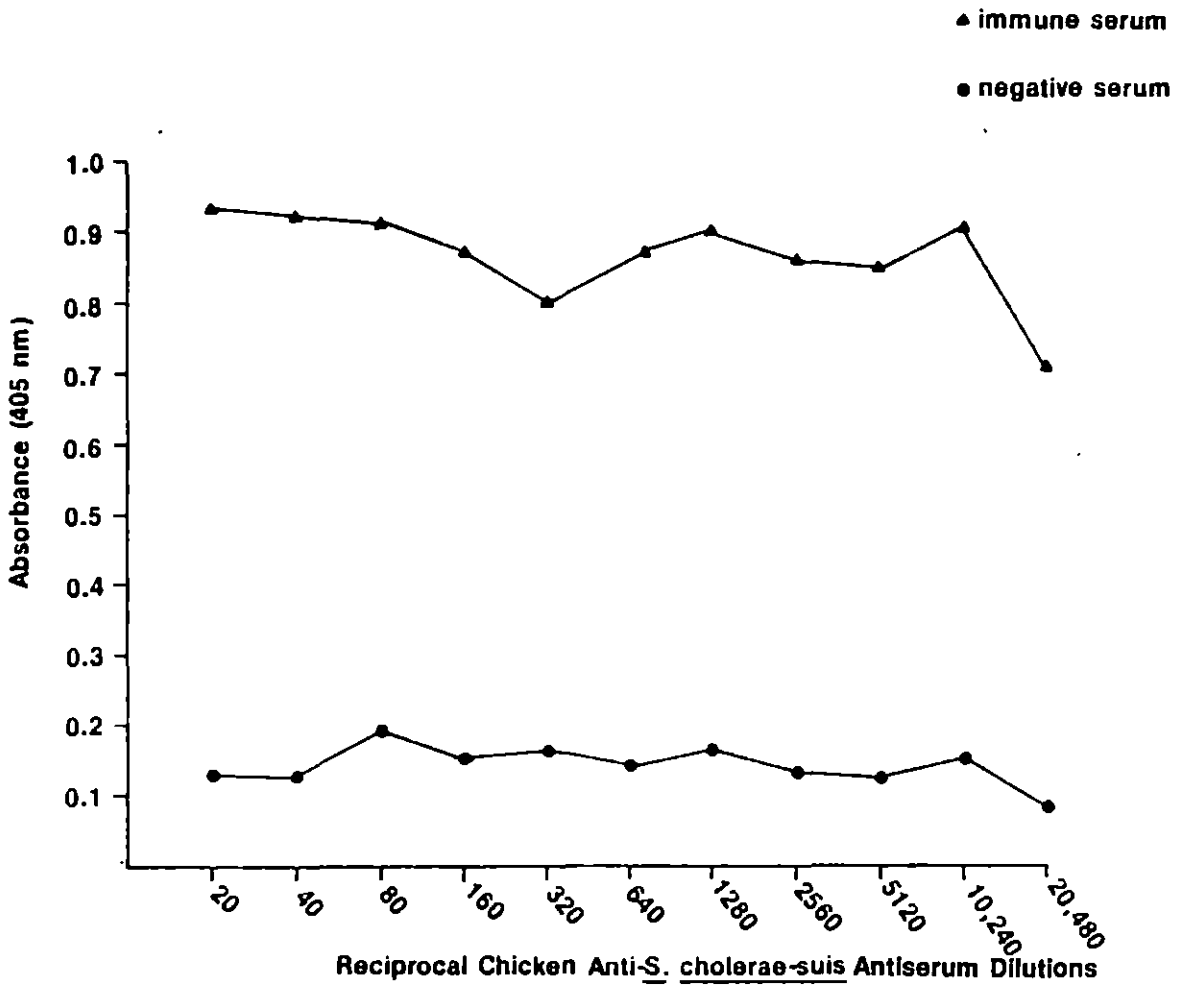


Figure 4. Modified Double Antibody Sandwich ELISA: titration of coating antibody, chicken anti-S. cholerae-suis. FT antigen diluted 1:500 was added to the plate coated with varying antibody dilutions, followed by 1:100 rabbit anti-S. cholerae-suis and 1:200 goat anti-rabbit peroxidase. Points represent the means of duplicate values. The graph indicates the optimum coating antibody dilution to be 1:5000, or the highest dilution displaying the highest specific and lowest non-specific reaction.

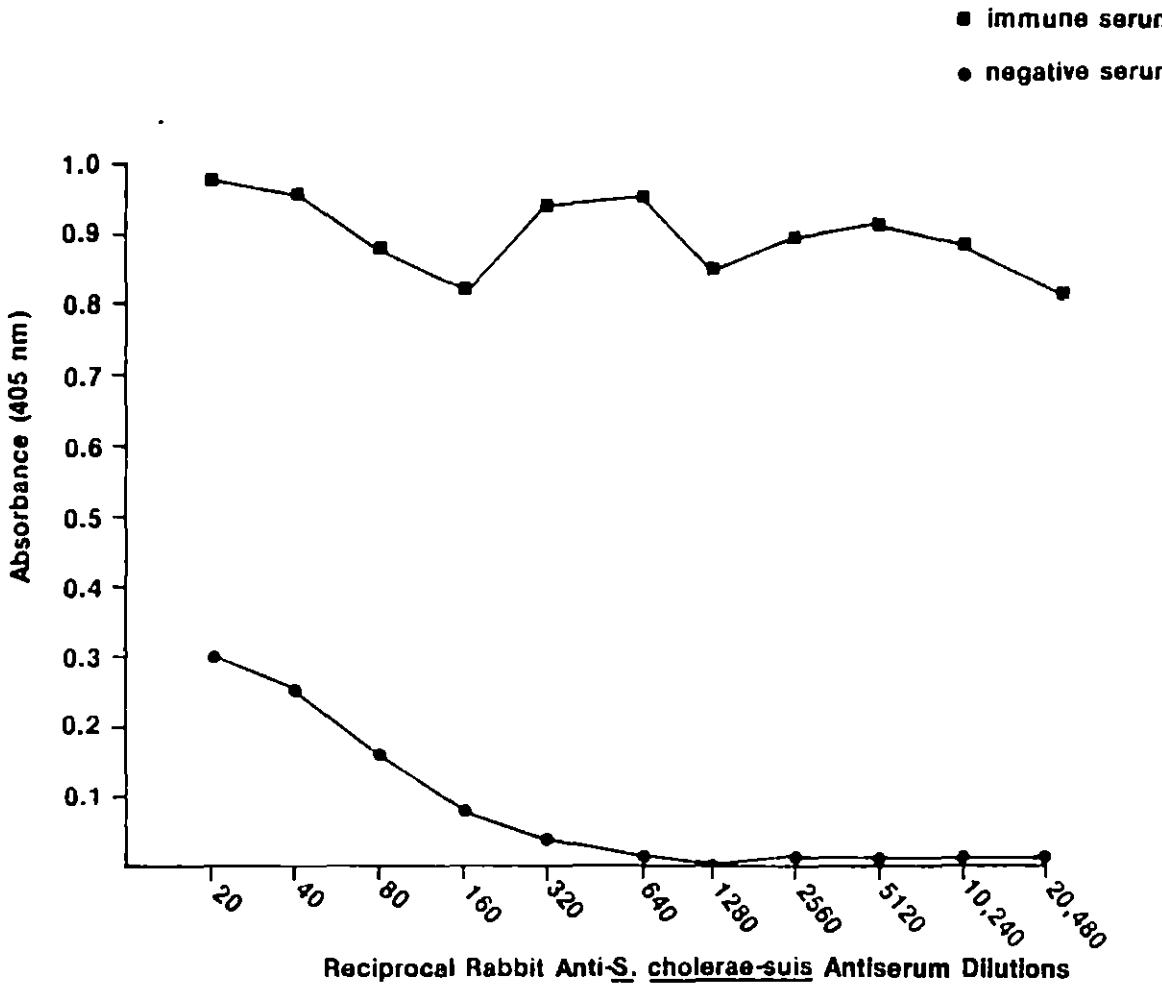


Figure 5. Modified Double Antibody Sandwich ELISA: titration of second antibody, rabbit anti-S. cholerae-suis. FT antigen diluted 1:500 was added to the plate coated with 1:5000 chicken anti-S. cholerae-suis. Goat anti-rabbit peroxidase was added at a 1:200 dilution. Points represent the means of duplicate values. The graph indicates the optimum second antibody dilution to be 1:5000, interpreted as in Fig. 4.

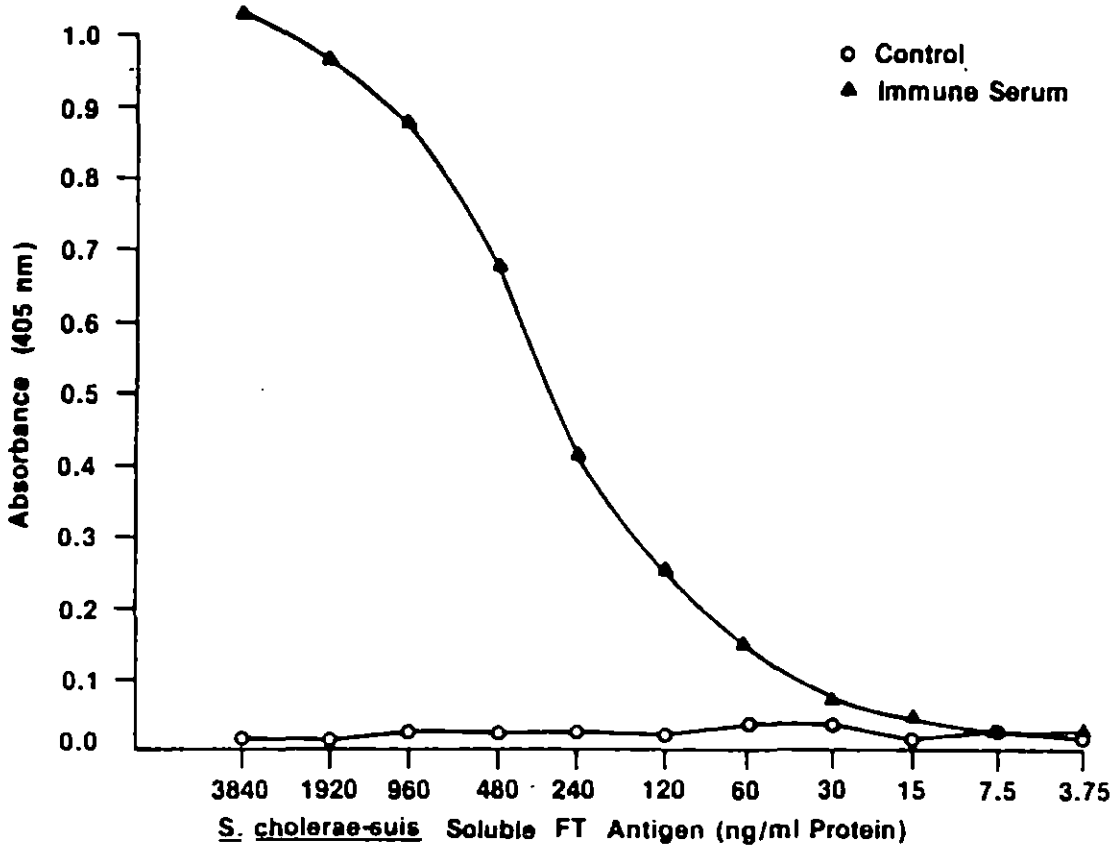


Figure 6. Modified Double Antibody Sandwich ELISA: limiting sensitivity to detect S. cholerae-suis FT antigen. FT antigen in various dilutions was added to the plate coated with 1:5000 chicken anti-S. cholerae-suis. Second antibody was added at a 1:5000 dilution and goat anti-rabbit peroxidase at 1:200. Points represent the means of duplicate values. The graph indicates the lowest concentration of FT detectable by this method to be 60 ng/ml, or the last point with a specific reaction above O.D. 0.1.

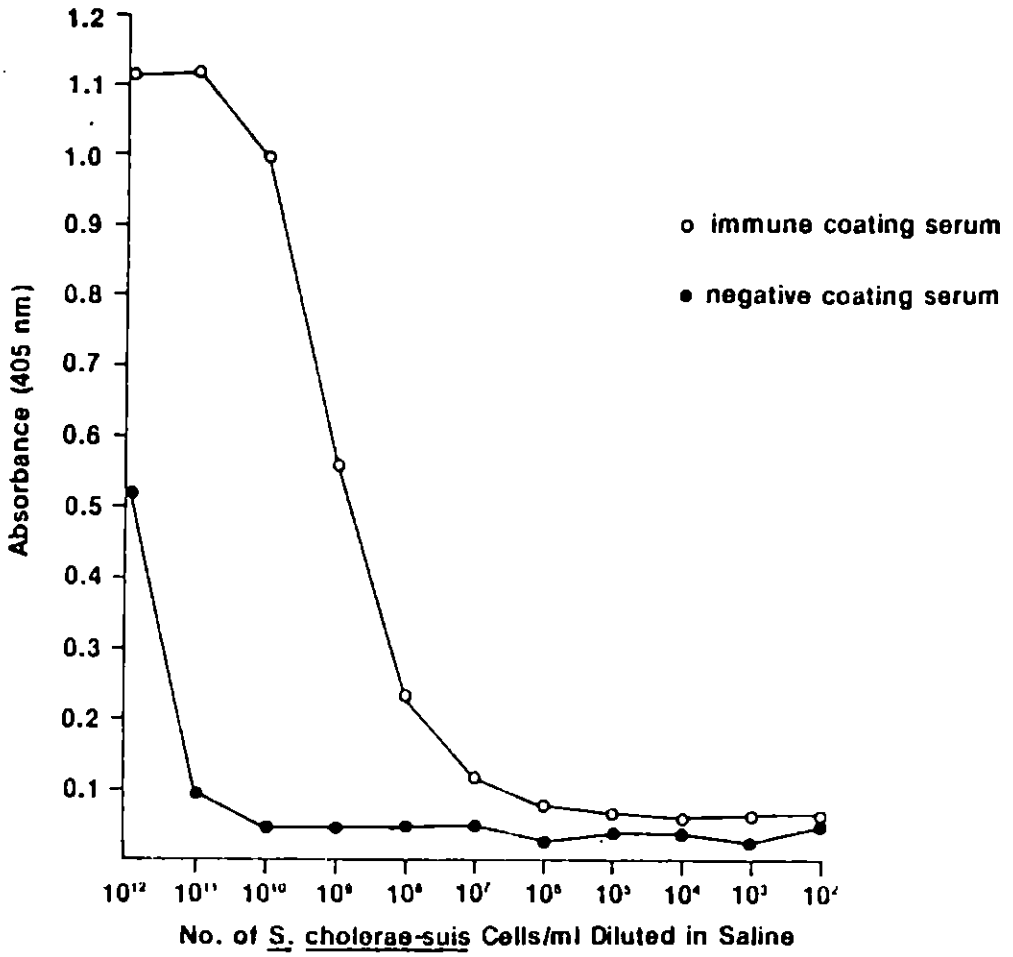


Figure 7. Modified Double Antibody Sandwich ELISA: limiting sensitivity to detect whole S. cholerae-suis cells. Various dilutions of whole cells were added to the plate coated with 1:5000 chicken antibody. Second antibody was then added at 1:5000 followed by goat anti-rabbit peroxidase at 1:200. Points represent the means of duplicate values. The graph indicates the lowest concentration of whole S. cholerae-suis cells detectable by this method to be 10^7 cells/ml, interpreted as in Fig. 6.

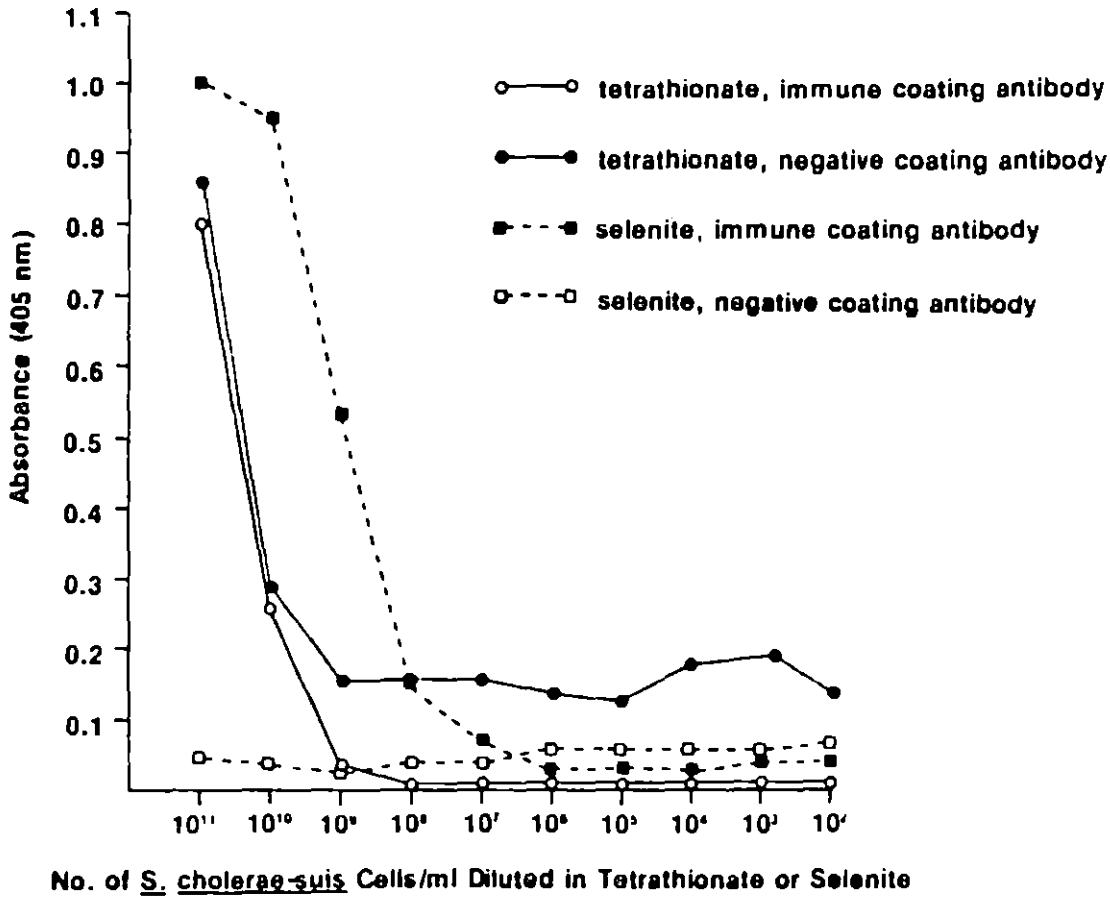


Figure 8. Modified Double Antibody Sandwich ELISA: detection of whole S. cholerae-suis cell in tetrathionate and selenite enrichment broths. Points represent the means of duplicate values. Note the high background readings with tetrathionate broth. Selenite broth allowed the detection of 10^8 cells/ml, interpreted as in Fig. 6-7.

PART III.

APPLICATION OF THE PEROXIDASE-ANTIPEROXIDASE IMMUNOASSAY TO THE IDENTIFICATION OF *SALMONELLAE* FROM PURE CULTURE AND ANIMAL TISSUE

Summary

The peroxidase-antiperoxidase immunoassay was developed by using selected *Salmonella* serotypes to evaluate its potential for use in diagnostic bacteriology. *S. cholerae-suis* var. *kunzendorf*, *S. dublin*, and *S. typhimurium* were the test organisms. Strong specific staining with corresponding antiserum was achieved with smears of each *Salmonella* serotype on microscope slides from formalinized cell suspensions, live cultures of clinical isolates, and tissue suspensions from the livers and spleens of experimentally infected mice. In addition, *S. cholerae-suis* var. *kunzendorf* was detected in formalin-fixed and fresh frozen tissues from experimentally infected pigs. The results of this study indicate that the peroxidase-antiperoxidase assay is well-suited for the rapid identification of *Salmonella* from pure cultures and that the technique can be useful in research for the detection of this pathogen in histological sections.

Introduction

The peroxidase-antiperoxidase immunoassay (PAP) is primarily a tool of histopathologists and is widely used for the demonstration of a variety of cell products in tissue sections. Detection of enzymes, polypeptide and steroid hormones, immunoglobulins, oncodevelopmental antigens, and viral antigens are documented applications of the PAP technique.[44] Sternberger in 1970 described the technique for the identification of Treponema pallidum in experimentally infected rabbit tissue.[156] Little information has since been published on the further development of this test for bacterial identification.[143,183]

The PAP method involves the sequential application of four basic reagents to the test antigen: primary antibody, secondary (anti-species) antibody, peroxidase-antiperoxidase of species origin identical to the primary antibody, and, finally, hydrogen peroxide combined with a suitable chromogen such as 3,3'-diaminobenzidine tetrahydrochloride (DAB).[27] The stained product with DAB is dark brown in color. Because the PAP method uses immunological rather than chemical bonding of peroxidase to antiperoxidase, difficulties encountered in other enzyme immunoassays utilizing labeled antibodies are overcome. These problems include destruction of antibody activity during the labeling process, introduction of background staining by the labeling process, and retention of unconjugated antibody.[154] In addition, sensitivity of the PAP method is reportedly greater than that of immunofluorescence,[156] the peroxidase-labeled antibody

sandwich method,[26] and radioimmunoassay.[119]

The purpose of this study was to apply the PAP procedure to the identification of some commonly encountered bacterial pathogens in human and animal medicine and to evaluate its potential for routine diagnostic use. Salmonella cholerae-suis var. kunzendorf, S. dublin, and S. typhimurium were used as test organisms. These 3 Salmonella serotypes were chosen because of their antigenic mosaics (Table 1): each serotype represents a different serogroup in the Kauffmann-White scheme.[106] Although S. typhimurium and S. dublin do share O antigens 1 and 12, the PAP method might allow differentiation between the two serotypes when reagents are optimally diluted. The development of this test, therefore, required finding the dilutions and incubation times of reagents that yielded specificity but also adequate intensity of staining. Bacteria were specifically identified in formalinized cell suspensions, live broth cultures of clinical isolates, and in tissue suspensions from the livers and spleens of experimentally infected mice. In addition, S. cholerae-suis was detected via this technique in formalin-fixed, paraffin-embedded, and fresh frozen tissues from experimentally infected pigs.

Table 1: Antigenic composition of selected Salmonella serotypes

Serotype	Group	Antigens
S. cholerae-suis var. kunzendorf	C_1	6,7:[c],1,5
S. dublin	D_1	1,9,12:g,p,-
S. typhimurium	B	1,4,[5],12:1,2

Bracketed antigens may be lacking.
Underlined antigens are present only when organism is lysogenized by converting bacteriophage.

Materials and Methods

Bacteria

Stock cultures of S. cholerae-suis var. kunzendorf and S. dublin were original field isolates that were stored in our laboratory in aliquots at -70C. A fresh isolate of S. typhimurium was obtained from the Iowa State University Veterinary Clinical Microbiology Laboratory, Ames. Bacteria were grown overnight in Trypticase soy broth (TSB, BBL Microbiology Systems), centrifuged, washed, and suspended in 0.5% formalinized saline to a density of McFarland tube no. 3. Live bacteria were maintained in TSB. A fresh animal isolate of Escherichia coli was likewise prepared for specificity testing.

Tissues

Three groups of five mice were injected intravenously with approximately 10^8 live S. cholerae-suis var. kunzendorf, S. dublin, or S. typhimurium organisms. Two animals in each group were selected for sacrifice at 48 hours postinjection on the basis of severity of observed clinical signs. Spleens and livers were removed and ground to a paste using Tenbroek grinders in 2-ml sterile saline. Smears of this paste were made on clean microscope slides. In addition, tissues were cultured for Salmonella in tetrathionate broth and subsequent plating on brilliant green agar. Serotypes of isolates from tissues were confirmed by plate agglutination, using commercial Salmonella typing sera (Fisher Diagnostics, Inc.).

Formalin-fixed and fresh frozen tissues from pigs experimentally infected with S. cholerae-suis var. kunzendorf were available from previous studies in our laboratory. These pigs were injected intratracheally with doses varying from 2.2×10^8 to 1.5×10^9 organisms in TSB and sacrificed 3 to 22 days postinfection. The PAP staining procedure as outlined below was applied to sections of lung, bronchial lymph node, liver, spleen, gall bladder, mesenteric lymph node, jejunum, ileocecal junction, colon, and cecum from 12 pigs, and selected tissues from an additional 14 pigs. Each tissue was cultured for S. cholerae-suis and identified as above.

Antisera

Primary antisera were produced separately in rabbits by intravenous injections with heat-killed suspensions of S. cholerae-suis var. kunzendorf and S. dublin. Rabbit origin anti-S. typhimurium antiserum was obtained commercially (Fisher). Secondary antiserum (goat anti-rabbit), normal goat serum, and PAP were also commercially produced (Polysciences).

Staining procedure

Tests were always performed in duplicate with appropriate controls. Slides were kept at room temperature and in a moisture chamber during staining. Reagents were applied as drops on top of the smear. After incubation with each reagent, excess reagent was gently shaken off and the slides were washed with 0.05M Tris (Sigma) saline

buffer (pH 7.6). Washing was accomplished by dipping the slides 25 to 30 times in buffer contained in staining dishes and then soaking for 5 min in a fresh sample of buffer. Excess moisture was then removed by blotting, but the slides were not allowed to dry completely before the next reagent was added.

Staining was accomplished in the following manner. Smears of cells or tissues were fixed in 10% methanol for 10 min.[154] For paraffin sections, staining was begun immediately after deparaffinization. Endogenous peroxidase activity was blocked by immersing the slides in a solution of 0.5% hydrogen peroxide in methanol for 10 min[159] for bacterial smears, or 0.075% acidified (HCl) methanol[61] for 30 min when staining paraffin or cryostat sections. Nonspecific background staining was then reduced by applying 3% normal goat serum in Tris buffer for 15 min.[156] Slides were blotted but not washed. Primary antiserum, rabbit anti-Salmonella, was added at an optimal dilution of 1:1000 (or 1:100 for commercial anti-S. typhimurium) for 15 to 30 min. Slides were washed and blotted. Secondary antiserum, goat anti-rabbit, was then applied for 15 min at a 1:50 dilution. Slides were again washed and blotted. The conjugate, optimally diluted rabbit PAP (1:50), was then added for 15 min. Slides were washed and blotted. The final step was the addition of a freshly prepared solution of 0.05% DAB and 0.01% hydrogen peroxide in Tris buffer for 5 to 8 min. Slides were washed in distilled water and counterstained, if appropriate.

Specificity of the test was determined on slides divided into

four sections with a diamond-point pencil. S. cholerae-suis var. kunzendorf, S. dublin, S. typhimurium, and E. coli were applied from formalinized preparations. Controls devoid of each reagent were included. When strong specific staining occurred, the presence of unstained bacteria of different Salmonella serogroups or E. coli was proven by application of dilute crystal violet for 15 to 30 sec, which did not interfere with the brown DAB stain. Specificity was also tested on the smears of murine liver and spleen tissue. This was accomplished by adding antisera to the three serotypes in optimal dilutions separately to three different smears from the same tissue, followed by the rest of the staining procedure. Tissue smears and fixed tissues were lightly counterstained with Giemsa as follows: Jenner's Working Solution, 5 min; Giemsa, 30 min; and 1% acetic acid, 1 to 1.5 min.

Results

PAP-stained Salmonella appeared swollen and outlined by the brown DAB stain (Fig. 1). PAP-stained cells were increased in size two to three times as compared to Gram-stained cells, similar to the classical "Quellung" reaction.

By the use of dilutions and incubation times of reagents as outlined in Materials and Methods, strong specific staining of each Salmonella serotype with corresponding antiserum was accomplished from live broth cultures, formalinized cell suspensions, and tissue

suspensions from experimentally infected mice (Fig. 3). In each case, the nonspecific serotypes (Fig. 2) and E. coli did not stain. PAP results matched culture results from the murine tissue suspensions.

Examples of S. cholerae-suis var. kunzendorf staining were detected in at least one of each of the sections of the lung, bronchial lymph node, liver, spleen, gall bladder, mesenteric lymph node, ileocecal junction, cecum, and colon from the 26 pigs (Table 2). Sections were scored from 1 to 4 for the appearance of PAP-stained Salmonella. A score of 0 was negative, 1 and 2 were questionable, and 3 and 4 were positive. The amount of background staining was similarly scored, ranging from 0 (no background) to 4 (high background). Bacteria were detected in both paraffin and cryostat sections. Except in the cecum, where Salmonella organisms were numerous and widespread in the lumen and intestinal glands (Fig. 4), the organisms, when stained, were few and widely scattered throughout the tissue sections, usually with no particular pattern. In the lung, they were seen free within the alveoli (Fig. 4) or closely associated with occluded blood vessels. Otherwise, the bacteria were not present in association with obvious histological lesions (Fig. 5). PAP results displayed little correlation to culture results.

Discussion

Salmonella serotypes were chosen for this study because of the amount of information on their antigenic relationships, the availability of cultures, information from previous studies in our laboratory, and the convenience of commercially prepared antisera. Because the test was made specific among serotypes of Salmonella, we believe that the technique can be applied to the identification of many other bacteria. Ultimately, a specific antiserum could be produced against any bacterial species or strain to be tested, similar to the widespread use of PAP for the diagnosis of many different carcinomas in tissue sections.[44] The test can be applied to pure cultures, clinical specimens, or tissues. The technique could be most helpful in identifying those organisms difficult to cultivate on artificial media, those difficult to identify by conventional biochemical tests, or bacteria usually identified by serological means, e.g., Salmonellae, Streptococcus, Leptospira, and E. coli. Thus, the test could be of use for many of the same reasons immunofluorescence is used in many laboratories today, except that the PAP method is more advantageous. It is reportedly 1,000 times more sensitive,[156] and the final colored product is stable and visible under light microscopy. Many slides can be run at one time, requiring approximately 2 hours for specific identification versus 2 to 4 days for biochemical identification. The cost of the test compares

favorably with conventional biochemical identification and provides a more rapid diagnosis. Thus, we believe the PAP technique has great potential for the identification of bacteria from pure cultures or clinical specimens in diagnostic laboratories.

This study also demonstrated the ability of the PAP technique to detect bacteria in fixed tissue. The poor correlation between culture and PAP results in tissue sections shown in Table 2 precludes the use of the technique for diagnostic purposes, at least with S. cholerae-suis. Also, the analysis of sections is time-consuming and inconsistent from section to section. But the association of bacteria with host tissue is a valuable attribute since information on the pathogenesis of the disease can result. In this study, Salmonella cells stained by PAP appeared for the most part in inaltered tissue and were never seen in association with severe lesions, such as lung consolidation and paratyphoid nodules in the liver. This may suggest damage to the tissue occurs via products, such as endotoxin, which result from the digestion and degradation of bacteria by the cell-mediated immune system. The relative absence of S. cholerae-suis from histological lesions agrees with the work of Lawson and Dow[104] who studied the pathology of this disease and detected the organisms via immunofluorescence.

Cryostat sections were slightly more effective than paraffin sections in detecting S. cholerae-suis using the PAP method. This is probably due to better preservation of antigens. However, the convenience and preservation of tissue morphology make paraffin

sections the best choice in studies of this type.

The major advantage of this application of the PAP method for identification is its proof of specificity and clear definition of the stained product (Fig. 1-5). Specificity is easily overlooked or difficult to achieve in studies utilizing immunoperoxidase and immunofluorescence. Non-specific staining is often problematic because it interferes with the determination of positive staining versus background staining.[135] Methods for inactivating endogenous peroxidase cannot eliminate 100% of the nonspecific staining. We found granules of non-specific brown stain in our sections, but this was easily differentiated from the characteristic morphology of the PAP-stained S. cholerae-suis.

Although the PAP-staining of S. cholerae-suis in porcine tissues did not correlate well with culture, the potential of the technique for the identification of a variety of other bacterial pathogens in tissues should be investigated. The technique is best-suited at this time for the rapid identification of pure cultures in diagnostic bacteriology.

Table 2: PAP and culture results for paraffin and cryostat sections from the tissues of pigs experimentally infected with S. cholerae-suis var. kunzendorf

Pig No.	Tissue	Days p.i.	culture	Paraffin		Cryostat	
				PAP	background	PAP	background
93	lung	5	+	0	1	0	1
	liver		+	0	1	0	0
	spleen		+	0	1	0	0
	mes. l.n.		+	0	1	0	1
	gall bl.		-	ND[a]	ND	0	0
	jejunum		+	0	1	0	0
	ileocecal		+	0	1	2	1
	cecum		+	0	1	4	1
	colon		+	0	1	3	1
	br. l.n.		+	ND	ND	3	1
94	lung	7	+	0	1	0	3
	liver		+	0	1	0	1
	spleen		+	0	1	0	1
	mes. l.n.		+	0	1	0	1
	gall bl.		+	ND	ND	0	2
	jejunum		+	0	1	0	1
	ileocecal		+	0	1	2	2
	cecum		-	0	1	0	1
	colon		-	0	1	3	1
	br. l.n.		+	0	1	0	1
97	lung	14	+	0	1	0	1
	liver		+	0	1	0	1
	spleen		+	0	1	0	1
	mes. l.n.		+	0	1	0	1
	gall bl.		-	ND	ND	2	1
	jejunum		-	0	1	0	1
	ileocecal		+	0	1	0	1
	cecum		-	0	1	0	1
	colon		-	1	1	0	1
	br. l.n.		+	ND	ND	0	1
99	lung	3	+	0	1	2	1
	liver		+	0	1	0	1
	spleen		+	0	1	0	1
	mes. l.n.		+	0	1	0	1
	gall bl.		-	ND	ND	0	1

[a] ND=not determined, due to unavailability of the sample.

Table 2: (continued)

Pig.No.	Tissue	Days p.i.	culture	Paraffin PAP	Paraffin background	Cryostat PAP	Cryostat background
99	jejunum		+	0	1	0	1
	ileocecal		+	0	1	0	1
	cecum		+	0	1	4	3
	colon		+	0	1	2	1
	br. l.n.		-	ND	ND	0	1
100	lung	5	-	0	1	0	1
	liver		-	0	1	0	1
	spleen		-	0	1	0	1
	mes. l.n.		-	0	1	0	1
	gall bl.		-	ND	ND	2	2
	jejunum		-	0	3	0	1
	ileocecal		-	0	1	0	1
	cecum		-	0	4	0	1
	colon		-	0	1	0	1
	br. l.n.		-	ND	ND	0	1
420	lung	3	+	0	1	0	1
	liver		-	0	2	0	1
	spleen		-	4	1	0	1
	mes. l.n.		+	0	1	0	1
	gall bl.		-	ND	ND	0	1
	jejunum		+	1	2	0	1
	ileocecal		+	0	1	4	1
	cecum		+	0	1	0	1
	colon		-	0	1	3	1
	br. l.n.		+	ND	ND	0	1
422	lung	7	-	0	1	0	1
	liver		-	0	1	1	1
	spleen		-	0	1	0	1
	mes. l.n.		+	0	1	1	1
	gall bl.		-	ND	ND	1	2
	jejunum		-	0	1	0	1
	ileocecal		-	0	3	0	1
	cecum		-	2	1	0	1
	colon		-	0	4	2	1
	br. l.n.		-	ND	ND	0	1
425	lung	3	+	0	2	1	2
	liver		+	0	2	0	2
	spleen		-	0	2	0	1
	mes. l.n.		+	0	1	2	1

Table 2: (continued)

Pig.No.	Tissue	Days p.i.	culture	Paraffin PAP	Paraffin background	Cryostat PAP	Cryostat background
425	gall bl.		-	ND	ND	4	1
	jejunum		+	0	1	0	1
	ileocecal		-	0	1	0	1
	cecum		-	0	1	0	1
	colon		-	0	2	0	1
	br.l.n.		-	ND	ND	1	1
429	lung	5	+	0	1	0	1
	liver		+	0	1	0	1
	spleen		+	0	1	0	1
	mes. l.n.		+	0	1	0	2
	gall bl.		-	ND	ND	0	1
	jejunum		+	0	1	0	1
	ileocecal		+	0	1	2	1
	cecum		+	2	1	3	1
	colon		+	1	1	3	1
	br. l.n.		-	ND	ND	2	1
431	lung	7	+	3	1	1	2
	liver		+	0	1	0	2
	spleen		+	0	1	0	1
	mes. l.n.		+	3	2	0	1
	gall bl.		+	ND	ND	0	1
	jejunum		+	1	1	0	1
	ileocecal		-	1	1	1	1
	cecum		-	0	1	0	1
	colon		-	0	1	0	1
	br. l.n.		+	ND	ND	0	1
432	lung	5	-	2	1	0	2
	liver		-	0	1	0	1
	spleen		-	0	4	4	1
	mes. l.n.		+	0	1	0	1
	gall bl.		-	ND	ND	0	1
	jejunum		-	0	2	0	1
	ileocecal		+	0	2	0	1
	cecum		+	0	1	4	1
	colon		-	0	1	3	1
	br. l.n.		+	ND	ND	2	1
532	lung	9	-	ND	ND	0	1
	liver		-			0	1
	spleen		-			0	1

Table 2: (continued)

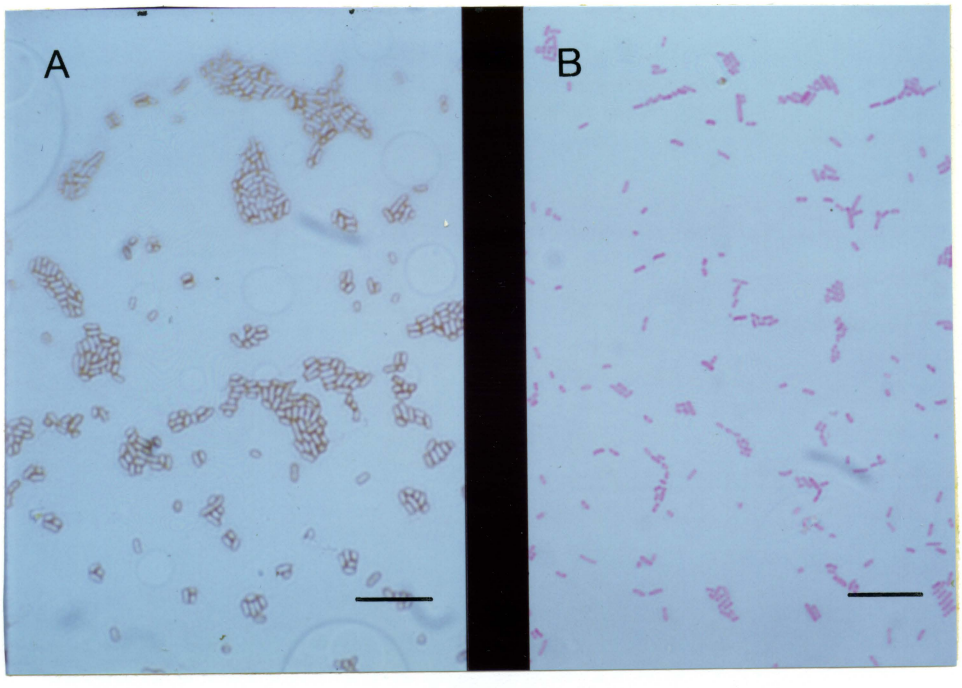
Pig.No.	Tissue	Days p.i.	culture	Paraffin PAP	Paraffin background	Cryostat PAP	Cryostat background
532	mes. l.n.		+	ND	ND	3	1
	gall bl.		-			0	1
	jejunum		-			0	1
	ileocecal		+			0	1
	cecum		+			0	1
	colon		-			0	1
	br. l.n.		+			0	1
2	lung	6	+	4	1	ND	ND
	liver		+	0	2		
	spleen		+	2	1		
7	lung	3	+	3	1	ND	ND
	liver		-	3	1		
	spleen		+	2	1		
	mes. l.n.		+	1	1		
11	lung	8	+	2	2	ND	ND
	liver		+	0	1		
	spleen		-	3	2		
	mes. l.n.		+	2	1		
19	lung	18	-	0	1	ND	ND
	liver		-	0	1		
33	lung	15	-	0	1	ND	ND
	liver		-	0	1		
	spleen		-	0	1		
	mes. l.n.		-	0	1		
45	jejunum	11	-	0	1	ND	ND
	ilececal		+	0	2		
	cecum		+	0	2		
73	lung	22	-	0	1	ND	ND
	liver		-	0	1		

Table 2: (continued)

Pig.No.	Tissue	Days p.i.	culture	Paraffin PAP	Paraffin background	Cryostat PAP	Cryostat background
402	lung	8	+	0	1	ND	ND
	liver		+	0	1		
	spleen		+	0	1		
	mes. l.n.		+	0	1		
	ileocecal		+	1	3		
	cecum		-	1	3		
	colon		+	1	3		
403	lung	11	+	0	1	ND	ND
	liver		+	0	3		
	spleen		+	0	2		
	mes. l.n.		+	0	4		
	jejunum		-	1	2		
	ileocecal		+	1	2		
	cecum		+	1	3		
406	lung	8	+	0	1	ND	ND
	liver		+	1	3		
	spleen		+	2	2		
	mes. l.n.		+	1	3		
407	jejunum	6	-	0	2	ND	ND
	ileocecal		+	2	3		
	cecum		-	0	2		
411	lung	6	-	4	1	ND	ND
	liver		-	4	1		
	spleen		+	3	1		
	mes. l.n.		+	3	1		
414	lung	4	-	0	3	ND	ND
	liver		+	0	3		
	spleen		-	0	1		
	mes. l.n.		+	0	1		
417	lung	15	+	0	1	ND	ND
	liver		+	1	3		
	spleen		+	1	2		
	jejunum		-	0	2		
	ileocecal		+	1	2		
	cecum		+	1	2		
	colon		+	0	2		

Fig. 1: Comparison of PAP-stained S. cholerae-suis var. kunzendorf (A) and Gram-stained cells (B). Note the marked swelling and sausage-shaped morphology of the PAP-stained cells. Bars, 20 µm.

Fig. 2: Bacterial smear stained for S. cholerae-suis via the PAP method. S. dublin counterstained with a dilute crystal violet (arrows).


A
B

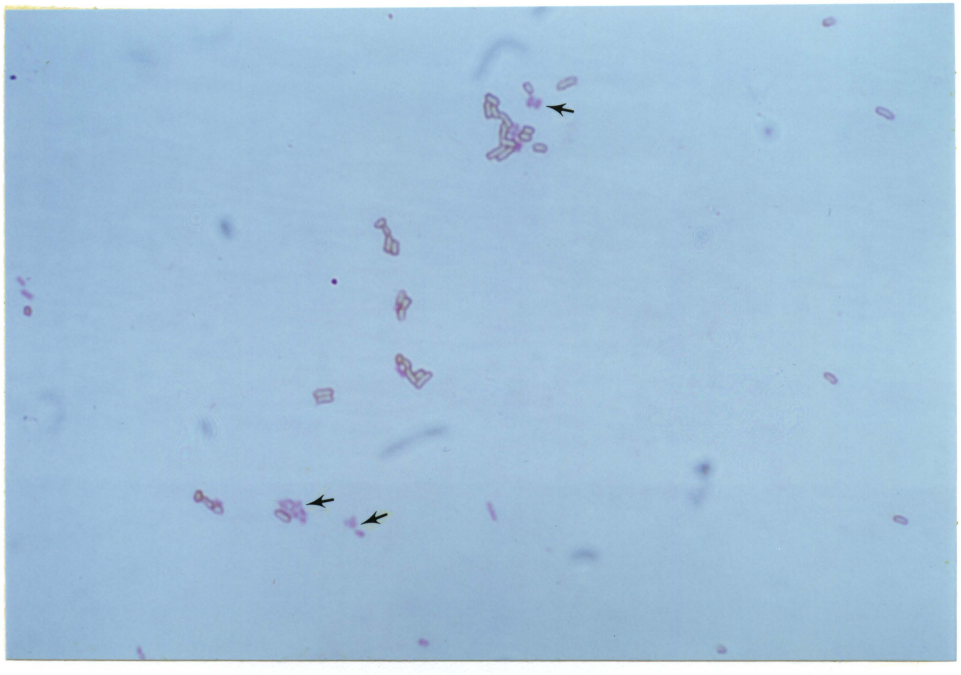


Fig. 3: PAP-stained S. typhimurium in a suspension of liver tissue from an experimentally infected mouse.

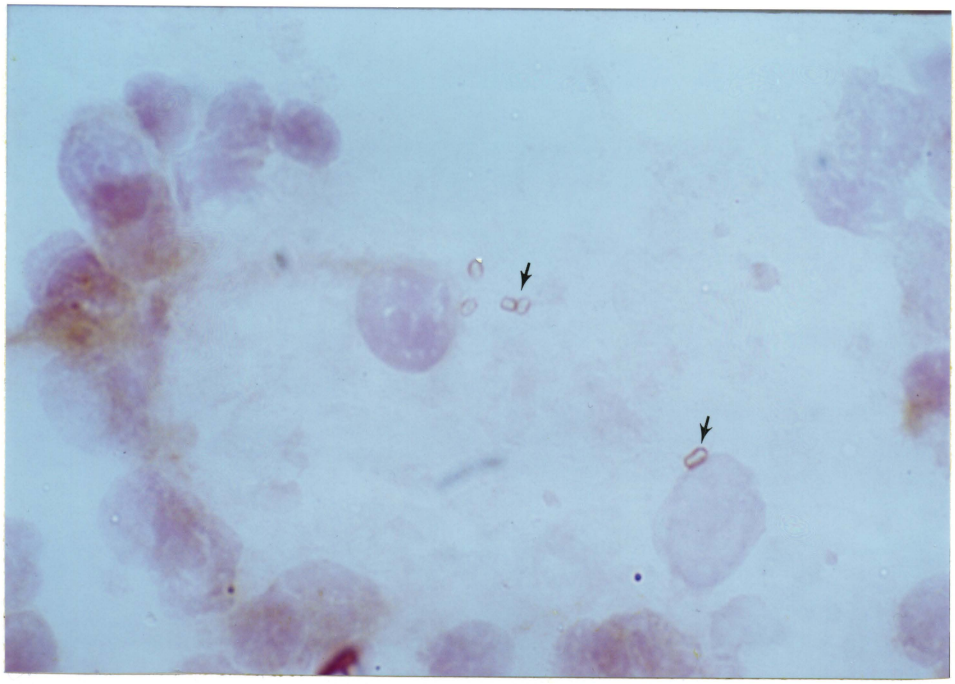
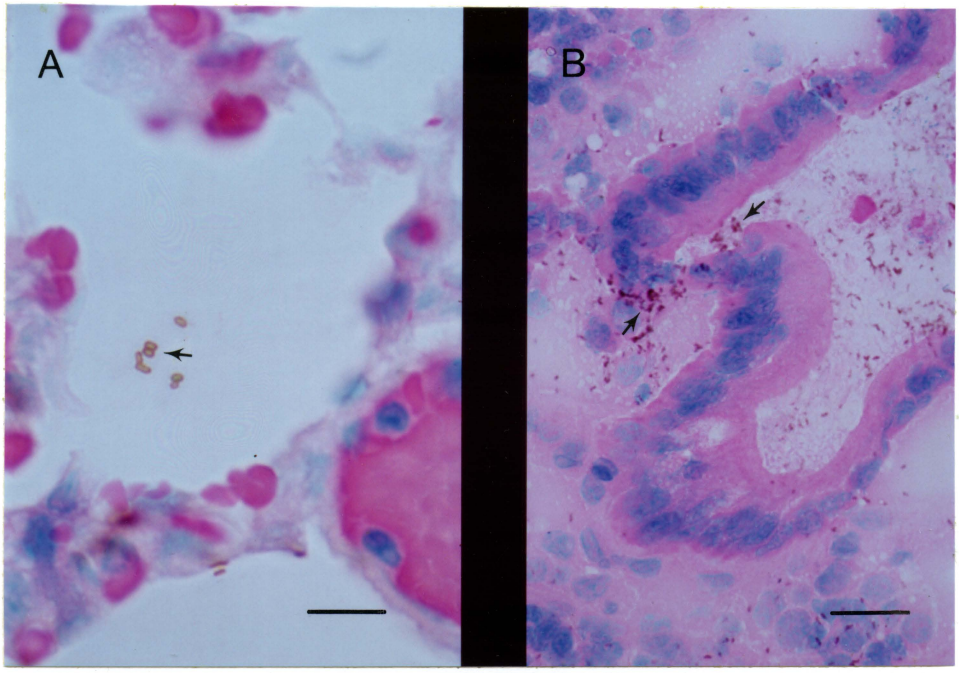
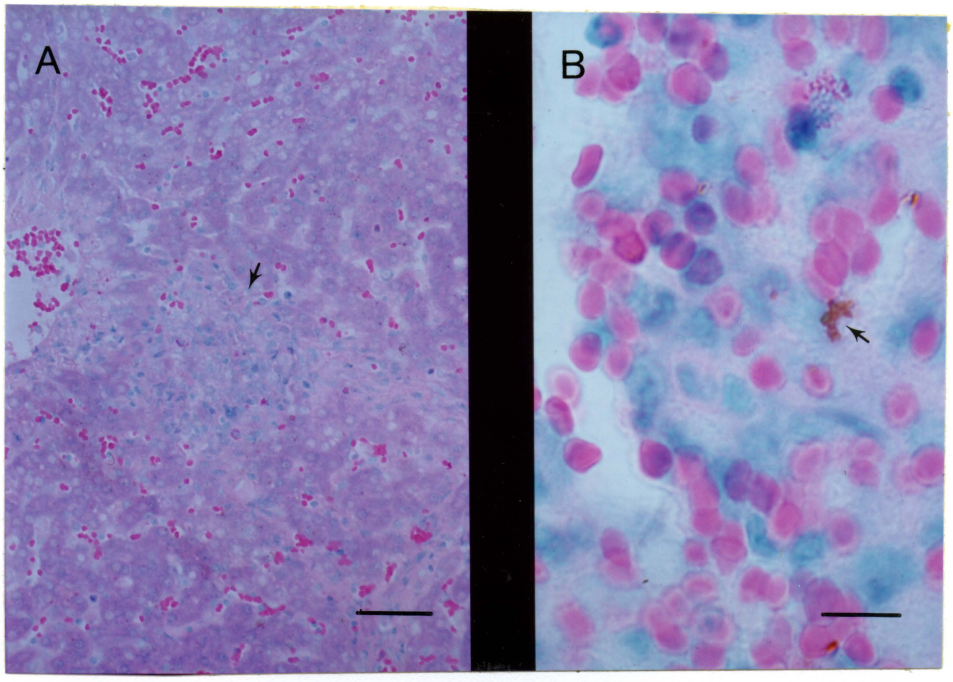


Fig. 4: (A) Formalin-fixed, paraffin-embedded porcine lung section stained for S. cholerae-suis (arrow) via the PAP technique and counterstained with a weak Giemsa (bar, 20 µm). (B) Cryostat section of a cecum stained stained in the same manner (bar, 40 µm). Arrows indicate stained S. cholerae-suis.

Fig 5: (A) Typical paratyphoid nodule in the liver of a pig (arrow), containing no Salmonella observable by the PAP technique (bar, 60 µm). (B) Microcolony of S. cholerae-suis in the same liver section (arrow), stained by PAP, in an area of the liver not containing a major histological lesion (bar, 20 µm).


A
B


A
B

SUMMARY

The results of this research indicate the suitability of Salmonella serotypes for application in a variety of immunoassays. The genus lends itself well to the development of immunoenzymatic tests because of its well-characterized antigenic composition, straightforward culture and identification procedures, and widespread clinical importance. The lengthy culturing procedure required to specifically identify Salmonellae from clinical specimens spawned this work to develop rapid techniques for identification. The objectives of this project were met by the application of the enzyme-linked immunosorbent assay (ELISA) and the peroxidase-antiperoxidase test (PAP) to the identification of Salmonella antibodies and antigens from experimentally infected animals, most importantly S. cholerae-suis var. kunzendorf in swine.

A successful indirect ELISA for the antibody responses of swine experimentally infected with S. cholerae-suis was developed. Pigs exhibited rising antibody levels to an extract of whole S. cholerae-suis cells from 1 to 4 weeks post-infection. ELISA titers were comparable to previously determined indirect hemagglutination titers, but the ELISA was a more rapid and convenient test to develop. The primary application for a test of this type, at least for swine salmonellosis, would be in research, since the serologic response of swine to Salmonellae has not been correlated with the carrier state. We found the test useful for detecting the antibody levels of sera

after treatment of swine with an immunosuppressant in the interest of elucidating immune mechanisms to this disease agent.

Asymptomatic swine carrying Salmonellae have been shown by other investigators to excrete the organisms in the feces during periods of stress, such as transport. These carrier animals are difficult if not impossible to detect using conventional culture methods. We therefore developed ELISAs for the detection of S. cholerae-suis antigen from the tissues and feces of experimentally infected pigs. Two types of assays were employed, an inhibition ELISA and a double-antibody sandwich ELISA, but only the latter exhibited potential for detecting the antigens of this organism in tissues. Although the sandwich ELISA detected as little as 60 ng/ml soluble protein antigen, it was not adequately sensitive to supplant routine bacterial culture for S. cholerae-suis in tissues and feces. However, the test was approximately 73-77% sensitive and 74-100% specific compared to bacterial culture and demonstrated the potential for improvement and subsequent application in the diagnostic laboratory or abattoir.

The PAP immunoassay also detected Salmonella antigens and allowed the visualization of the organisms on bacterial smears and histologic sections. The test was highly specific and could shorten the identification period for Salmonellae if applied to pure cultures. Detection of the organisms in formalin-fixed, paraffin-embedded and fresh frozen tissues did not correlate with culture results, but allowed association of the bacteria with host tissue. These findings demonstrated the potential of the PAP technique for the identification

of a variety of other bacterial pathogens difficult to detect via culture methods.

LITERATURE CITED

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