

Tissue distribution of enterotoxigenic Escherichia coli
K99 pilus antigen in pregnant swine following feeding
of a live oral vaccine

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by

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A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
MASTER OF SCIENCE

Major: Veterinary Pathology

Signatures have been redacted for privacy

Iowa State University
Ames, Iowa

1983

1461354

TABLE OF CONTENTS

	Page
INTRODUCTION	1
PART I. APPLICATION OF THE PEROXIDASE-ANTIPEROXIDASE TECHNIQUE FOR THE DETECTION OF ENTEROTOXIGENIC <u>ESCHERICHIA COLI</u> K99 PILUS ANTIGEN IN PARAFFIN-EMBEDDED TISSUES	2
INTRODUCTION	3
STATEMENT OF PROBLEM AND OBJECTIVES	9
MATERIALS AND METHODS	10
Animals and inoculation	10
Necropsy and specimen collection	10
Tissue processing	10
Treatment for endogenous peroxidase	12
Sera	12
Staining solution	13
Peroxidase-antiperoxidase staining technique	13
Specificity	15
Sensitivity	16
RESULTS	16
DISCUSSION	26
CONCLUSIONS	28
PART II. TISSUE DISTRIBUTION OF ENTEROTOXIGENIC <u>ESCHERICHIA COLI</u> K99 PILUS ANTIGEN IN PREGNANT SWINE FOLLOWING FEEDING OF A LIVE ORAL VACCINE	29
INTRODUCTION	30
STATEMENT OF PROBLEM AND OBJECTIVES	43
MATERIALS AND METHODS	44
Animals	44
Vaccines	44
Vaccination	44
Serum	45
Necropsy, specimen collection, and processing	45
Bacterial culture	47
Agglutination	48

	Page
RESULTS	49
DISCUSSION	63
CONCLUSIONS	67
LITERATURE CITED	69
ACKNOWLEDGMENTS	94

INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) in the small intestine of piglets produce a noninvasive infection that is characterized by a profuse secretory diarrhea. The net result is dehydration, acidosis due to the loss of electrolytes, and death.

Colonization of the small intestine by ETEC stimulates a vigorous secretory immune response principally involving immunoglobulin A. Furthermore, oral vaccination of pregnant swine with live ETEC leads to the appearance of specific antibodies in the colostrum (88, 106) and milk (49). Secretion of specific antibodies appears to be more prolonged in the milk than in the intestine of orally vaccinated swine (49).

Pilus antigens are an important protective component of ETEC when given in oral vaccines (106). In addition, live oral ETEC vaccines are more effective in provoking a secretory immune response than are killed ETEC (49, 106) or live rough non-ETEC (106).

The tissue distribution and persistence of live ETEC vaccines in orally vaccinated pregnant swine has not been investigated. Determining the distribution of both live ETEC and a weakly immunogenic strain of non-ETEC would enhance the understanding of secretory immunity in this species. This study has been undertaken to determine the distribution and persistence of two live oral vaccines, each bearing K99 pilus antigen. The peroxidase-antiperoxidase technique of Sternberger will be evaluated as a method of detecting K99 pilus antigen in selected tissues of orally vaccinated swine.

PART I: APPLICATION OF THE PEROXIDASE-ANTIPEROXIDASE TECHNIQUE FOR
THE DETECTION OF ENTEROTOXIGENIC ESCHERICHIA COLI K99 PILUS
ANTIGEN IN PARAFFIN-EMBEDDED TISSUES

INTRODUCTION

The identification and localization of antigens in tissues has become an invaluable technique in histochemistry and diagnostic pathology. Immunohistochemical methods employed today are, commonly, labeled antibody methods. Antibodies are chemically conjugated with fluorescent, electron dense, or enzymatic tracers. Any antigen against which a specific antibody can be prepared may be visualized with fluorescence, electron, or light microscopy.

Labeled antibody methods are, generally, direct or indirect staining procedures. The direct staining procedure is a one-step procedure in which labeled antibody specific for a particular antigen is employed.

The indirect localization procedure employs unlabeled specific antibody which binds to a particular antigen. This bound antibody is then visualized with a labeled antiglobulin specific for the gamma globulin (IgG) of the first antibody.

Indirect procedures eliminate the necessity to prepare labeled antibodies for each antigen to be localized. Additionally, because each antibody molecule binding to an antigen will itself bind several labeled antiglobulin molecules, fluorescence or enzymatic reaction product will be more visible than in direct procedures.

Historically, immunohistochemistry was introduced by Coons and colleagues who demonstrated that antibodies labeled with fluorescein isothiocyanate could combine with antigen in tissue and then be visualized with fluorescence microscopy (36).

The subsequent development of immunoperoxidase procedures in which the enzyme, horseradish peroxidase, was conjugated to antibody (5, 101, 113) has since been shown to be adaptable to both electron and light microscopy (114, 158).

There are potential problems inherent to labeled antibody methods. Covalent bonding to antibody has been shown to decrease antibody activity (158). In addition, nonspecific antibodies are labeled equally with specific antibodies which increases nonspecific staining (160).

The peroxidase-antiperoxidase (PAP) technique of Sternberger employs unlabeled antibodies to link the visualizable enzyme, horseradish peroxidase, to antigen in tissue (160). The antigen in question is first localized by diluted primary antiserum (first antibody). The application of the first antibody is followed by an excess of a second antibody directed against the first antibody species' IgG. In other words, if the first antibody is of rabbit origin, the second antibody could be goat anti-rabbit IgG.

The next step is the application of an affinity-purified, soluble PAP complex that consists of three molecules of horseradish peroxidase immunologically bound to two molecules of antiperoxidase. The antiperoxidase in the complex is from the same species as the first antibody, so it provides an antigen (IgG) capable of reacting with the free combining site of the second antibody. The peroxidase in the complex is therefore efficiently and irreversibly bound to the antigen localized by the first antibody.

Visualization of the attached complexes involves the use of hydrogen peroxide as enzyme substrate and, generally, diaminobenzidine as the

electron donor (57). The process leads to deposition of a golden brown, highly insoluble polymeric reaction product. The latter is osmiophilic and can be intensified (darkened) by exposure to fumes of 1% or 2% osmium tetroxide.

Other electron donors may be employed in the PAP procedure and labeled immunoperoxidase procedures. One electron donor is 4-chloro-1-naphthol, which generates a less insoluble blue reaction product. It is possible to stain one antigen in a tissue section brown with diaminobenzidine, and then repeat the staining procedure with an antibody to another antigen using 4-chloro-1-naphthol to obtain an immunospecific blue counterstain (112). In this way, two different antigens can be stained with different chromogens in the same tissue section.

The PAP technique is reportedly a very sensitive immunohistochemical procedure. It is characterized by low background staining due to the triple specificity immunologic amplification inherent in the method. When compared with labeled immunoperoxidase procedures, the PAP technique was shown to be 20-125 times more sensitive (25, 132). When compared with immunofluorescence procedures, the PAP technique was reported to be 100-1000 times more sensitive (159). It was also capable of detecting very small amounts of antigen (110).

One major disadvantage of the PAP technique and other immunoperoxidase procedures is that the presence of endogenous peroxidase activity in tissues can interfere with interpretation of the staining reaction. Consequently, endogenous peroxidase activity in many tissues must be inactivated prior to staining (80, 162, 163). Unfortunately, pretreating tissues to destroy endogenous peroxidase may result in the loss of some

antigenicity (33) and the effect of pretreatment on each antigen must be tested.

Staining of tissues for immunofluorescence and some immunoperoxidase procedures is commonly done on frozen tissue sections. The freezing and thawing of tissues does not significantly alter antigen reactivity; however, fine morphological detail is seldom preserved and diffusion of antigen may occur. In addition, long-term storage of stained sections and retrospective studies are not practical when frozen sections are utilized.

The purpose of tissue fixation is to arrest autolytic enzyme activity and to preserve morphological detail of the tissue. In immunohistochemistry, fixation also prevents the diffusion of antigen. The fixative employed must allow for retention of sufficient antigenic reactivity in the tissue, however.

Formaldehyde and other aldehyde-based fixatives induce the formation of methylene group linkages within and between adjacent peptide chains of protein (130). This extensive cross-linking results in the denaturation of protein and may sterically hinder or "mask" many protein antigens. Larger protein antigens whose reactivity does not only depend on primary structure, but also on conformational determinants, appear to be especially vulnerable to this effect (84).

Staining of immunoglobulins in formalin-fixed, paraffin-embedded tissues by labeled immunoperoxidase and immunofluorescence procedures has been reported (26, 43, 164). Other studies have demonstrated inconsistent results when these methods were applied to formalin-fixed, paraffin-embedded tissues (14, 93, 100).

Sternberger suggested using the sensitive PAP technique to overcome the adverse effects of aldehyde-based fixatives (159). Localization of viral antigen in formalin-fixed, paraffin-embedded tissues using the PAP method has been reported (46, 47, 56).

The use of various proteolytic enzymes to unmask antigen in aldehyde-fixed, paraffin-embedded tissues has been demonstrated (23, 42, 43, 70, 141, 142), although the exact mechanism of unmasking is unknown. Curran and Gregory suggested that antigen in aldehyde-fixed tissue may fail to react immunologically because of fixative-induced impermeability of the tissue and not because the fixative directly alters or destroys the antigen. They believe that proteolytic enzymes may "etch" the surface of the tissue section exposing previously masked antigenic determinants (42).

Temperature influences the rate at which aldehyde fixatives denature protein. In an early study, Seligman, Chauncey, and Nachlas fixed slices of rat liver in 10% neutral phosphate-buffered formalin for varying times at varying temperatures. The liver homogenates were then tested for activity of various enzymes. Formalin fixation at 25°C and 37°C denatured the majority of enzymes very rapidly. Fixation with cold (4°C) formalin resulted in a much smaller loss of enzyme activity initially. However, denaturation proceeded slowly when fixation was allowed to continue for 24 hours (148).

Alcohol fixatives such as ethanol and methanol fix tissue by precipitating proteins (130). The cold alcohol fixation and dehydration method of Sainte-Marie (146) has been shown to preserve antigenic reactivity of

immunoglobulins in paraffin-embedded tissue (26). This method may provide an attractive alternative to aldehyde-based fixation.

STATEMENT OF PROBLEM AND OBJECTIVES

Determining the distribution of enterotoxigenic Escherichia coli K99 pilus antigen after oral vaccination of pregnant swine would enhance the understanding of secretory immunity in this species. The PAP technique of Sternberger has been demonstrated to be a sensitive immunohistochemical method employed for localization and identification of antigen in tissue.

This study will be undertaken to evaluate the sensitivity, specificity, and feasibility of the PAP technique when employed to detect enterotoxigenic E. coli K99 pilus antigen in paraffin-embedded tissue of swine.

MATERIALS AND METHODS

Animals and inoculation

Colostrum-deprived piglets were procured from a conventionally farrowed swine herd maintained by the National Animal Disease Center (NADC), Ames, Iowa. Stationary-phase broth (Trypticase soy broth-BBL, Becton, Dickinson, Cockeysville, MD) cultures of enterotoxigenic E. coli (ETEC) strains 431 (0101:K30:K99:NM) and 987 (09:K103:987P:NM) were grown aerobically for 16 hours at 37°C. Each piglet was given 1 ml of either broth culture (10^8 - 10^9 viable bacteria) in 20 ml of fresh broth by gavage within the initial 6 hours of life. Both strains were originally isolated from intestines of piglets with diarrheal disease. One piglet was not inoculated.

Necropsy and specimen collection

Piglets were euthanized by an intracardial injection of pentobarbital (Pentobarbital Sodium, D-M Pharmaceuticals Inc., Rockville, MD) 18 hours after inoculation or at an earlier time if they were moribund. Sections of ileum were collected to be processed for PAP staining and evaluation with the light microscope. Tissues from piglets inoculated with strain 987 and the uninoculated piglet were processed to serve as tissue controls.

Tissue processing

Various combinations of fixatives and dehydrating alcohols, including formalin, ethanol, and methanol, were employed to determine which procedures would preserve the immunologic reactivity of K99 pilus

antigen. Trypsin digestion of formalin-fixed tissues (70) was also attempted. Two methods of fixation and dehydration preserved antigenic reactivity of K99 pilus antigen in paraffin-embedded tissue.

Modified method of Sainte-Marie All processing steps were conducted at refrigerator temperature (4°C) according to the method of Sainte-Marie (146), but with some variation. Sections of ileum 2-4 mm thick were fixed in cold 95% methanol for 20-22 hours. Dehydration was accomplished in four changes of cold absolute methanol for 1 hour each. Sections were cleared with frequent agitation in two changes of cold xylene for 1 hour each. The last change of xylene was allowed to come to room temperature. Prior to embedding, sections were processed through three changes of filtered paraffin (Paraplast, Lancer, St. Louis, MO) at 60°C for 1 hour, 1/2 hour and 1/2 hour respectively. After embedding, 5 μm tissue sections were cut and placed on clean glass slides. Cut sections were deparaffinized at room temperature in two changes of xylene for 1 minute each, two changes of absolute methanol for 1 minute each, two changes of 95% methanol for 1 minute each, and one change of 70% methanol for 1 minute. Prior to PAP staining, the tissue sections were rinsed in three changes of Tris-HCl (Trizma-HCl, Sigma Chemical Co., St. Louis, MO) buffer, pH 7.6, for 2 minutes each. Glass distilled water was employed as solvent for the buffer.

Cold formalin fixation, cold methanol dehydration All processing steps were conducted at refrigerator temperature (4°C). Sections of ileum were fixed in cold 10% neutral phosphate buffered formalin for 24 hours. The sections were dehydrated in two changes of cold 70% methanol for 1 hour each, two changes of cold 95% methanol for 1 hour each, and

three changes of cold absolute methanol for 1/2 hour each. Clearing of tissue sections required frequent agitation and was accomplished in two changes of cold xylene for 1 hour each. The last change of xylene was allowed to come to room temperature. Infiltration with paraffin, sectioning, deparaffinization, and rinsing of tissue sections was performed as previously described.

Treatment for endogenous peroxidase

Tissue sections were treated for 20 minutes with absolute methanol containing 1% sodium nitroferricyanide and 1% glacial acetic acid (162). Treatment was incorporated into the deparaffinization process immediately following the final change of absolute methanol and preceding the initial change of 95% methanol.

Sera

All sera, including control sera, were diluted in 0.05 M Tris-HCl buffer in glass distilled water, pH 7.6. One percent normal goat serum (Polysciences, Inc., Warrington, PA) was added to the diluent to reduce nonspecific staining. Rabbit K99 antiserum (absorbed standard 1:10) (108) was provided by the Colibacillosis Research Unit of NADC. A dilution of 1:100 was determined to be optimal because of reduced nonspecific staining. Goat anti-rabbit serum (Polysciences) diluted 1:50 and rabbit peroxidase-antiperoxidase (Polysciences) diluted 1:100 were determined to be optimal concentrations for PAP staining.

Staining solution

A 0.05% solution of diaminobenzidine (DAB) (3,3'-diaminobenzidine tetrahydrochloride, Sigma) in 0.05 M Tris-HCl buffer, pH 7.6, was prepared upon a magnetic stirring apparatus 30 minutes prior to staining. Undissolved DAB was removed by filtration with a .45 μm filter (Millex-HA, Millipore Corporation, Bedford, MA). Immediately before staining, 30% H_2O_2 was added to the filtrate so as to produce a final H_2O_2 concentration of 0.01%.

Peroxidase-antiperoxidase staining technique

All steps were carried out in a moist chamber (water bath) at room temperature. Except as noted, after each incubation, the tissue sections were washed in three changes of 0.05 M Tris-HCl buffer, pH 7.6, in glass distilled water. Each change lasted 3 minutes with continuous agitation provided by a magnetic stirring apparatus. The area around sections was blotted with filter paper to prevent running of subsequently applied solutions.

The procedure was performed as follows:

1. 20% normal goat serum diluted in Tris-HCl buffer was applied to the tissue sections for 30 minutes to reduce nonspecific staining.
2. The normal goat serum was decanted from the sections after incubation, but the sections were not washed.
3. Rabbit K99 antiserum (1:100) was applied to the sections for 30 minutes.
4. Sections were washed 3X in Tris-HCl buffer.

5. Goat anti-rabbit serum (1:50) was applied to the sections for 30 minutes.
6. Sections were washed 3X in Tris-HCl buffer.
7. Rabbit peroxidase-antiperoxidase (1:100) was applied to the sections for 30 minutes.
8. Sections were washed 3X in Tris-HCl buffer.
9. Sections were placed in a staining dish containing the freshly prepared DAB-H₂O₂ staining solution for 5-8 minutes, but no longer than 8 minutes.
10. The staining reaction was stopped by placing the tissue sections in distilled water.

Following all staining procedures, tissue sections were washed in three changes of distilled water for 2 minutes each, counterstained with Giemsa, dehydrated, cleared, mounted, and observed under the light microscope. Sections fixed in cold formalin and dehydrated in cold methanol were exposed to the fumes of 1% osmium tetroxide for 1 minute after the staining reaction was stopped. The sections were then washed and counterstained with Giemsa. All tissue sections were well differentiated in .5% acetic acid during the Giemsa staining process.

Controls Control sera and colostrum were obtained from the Colibacillosis Research Unit of NADC. Rabbit K88 antiserum (absorbed standard 1:10) (147) and normal rabbit serum (NRS) were diluted 1:100 and substituted for rabbit K99 antiserum in the staining procedure. These two sera served as primary serum controls. Swine colostrum with K99 antibody (1:6400) (106) diluted 1:20 was applied to sections for 30 minutes, and then the sections were washed prior to PAP staining in an

attempt to "block" the staining reaction. Sections from piglets inoculated with ETEC strain 987 and from the uninoculated (normal) piglet served as tissue controls.

Specificity

A variety of controlled tests were employed to evaluate specificity of the PAP technique for K99 pilus antigen in paraffin-embedded tissue. All tests were conducted by a technician. All test sera and tissues were encoded by an individual other than the one conducting the tests. Test results were evaluated by an unbiased observer. Test sera and tissues were decoded following completion of the tests. The PAP staining procedure, employing rabbit K99 antiserum, was performed on normal piglet tissue and tissues with K99 and 987P pilus antigens. NRS, rabbit K88 and rabbit K99 antisera were employed as primary sera on tissue with K99 pilus antigen. Swine colostrum with K99 antibody and NRS were employed as blocking reagents prior to PAP staining on tissue with K99 pilus antigen.

Sensitivity

The ability of the PAP technique to detect a minute quantity of ETEC K99 pilus antigen in tissue was evaluated. An ileocolic lymph node containing $3.5 \log_{10}$ colony-forming units of ETEC strain 1459 (09:K35:K99:NM)/gm tissue was obtained from an orally vaccinated gilt in a pilot study (Part II). The tissue was fixed in cold 95% methanol and processed for PAP staining. Appropriate controls were implemented and the sections were evaluated.

RESULTS

EPEC strain 431 was most clearly and precisely identified on villous surfaces in the cold methanol processed tissues (Figures 1.1 and 1.2). Reaction product in these tissues did not require intensification with osmium. Staining intensity was somewhat diminished in tissues fixed in cold formalin. Exposure of these tissue sections to the fumes of 1% osmium tetroxide was required to enhance visualization of reaction product (Figures 1.3 and 1.4). Intensification with osmium often darkened the entire tissue section and obscured fine morphological detail. Both methods of fixation and processing adequately preserved tissue morphology.

The endogenous peroxidase activity of leukocytes and erythrocytes was destroyed when piglet tissue sections were treated with absolute methanol containing 1% sodium nitroferricyanide and 1% glacial acetic acid. Treatment of sections for longer than 25 minutes decreased antigenic reactivity of K99 pilus antigen.

Nonspecific staining of tissue sections was occasionally encountered, making interpretation difficult. Applying higher concentrations of normal goat serum prior to staining did not decrease nonspecific binding of antibody, and in most cases, decreased staining intensity.

The PAP technique was specific for K99 pilus antigen (Table 1.1). Normal piglet tissue and tissue containing 987P pilus antigen were not stained when rabbit K99 antiserum was employed in the staining procedure (Figures 1.5 and 1.6). NRS and rabbit K88 antiserum, when substituted as primary sera, did not stain tissue containing K99 pilus antigen.

Figure 1.1. Piglet ileum processed in cold methanol according to a modified method of Sainte-Marie. ETEC strain 431 colonizing villi is identified by brown reaction product (arrow).

Well-differentiated Giemsa stain

Figure 1.2. Magnification of section in Figure 1.1

Figure 1.3. Piglet ileum fixed in cold formalin, dehydrated in cold methanol, and post-fixed in 1% osmium tetroxide. ETEC strain 431 colonizing villi is identified by brown reaction product (arrow).

Giemsa stain

Figure 1.4. Magnification of piglet ileum fixed in cold formalin, dehydrated in cold methanol, and post-fixed in 1% osmium tetroxide. ETEC strain 431 colonizing villi is identified by brown reaction product (arrow). Giemsa stain

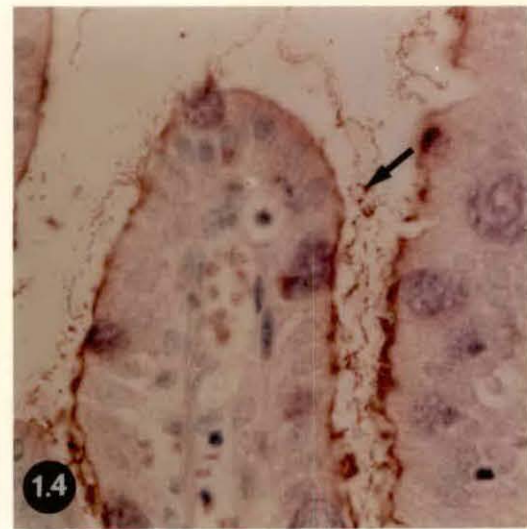
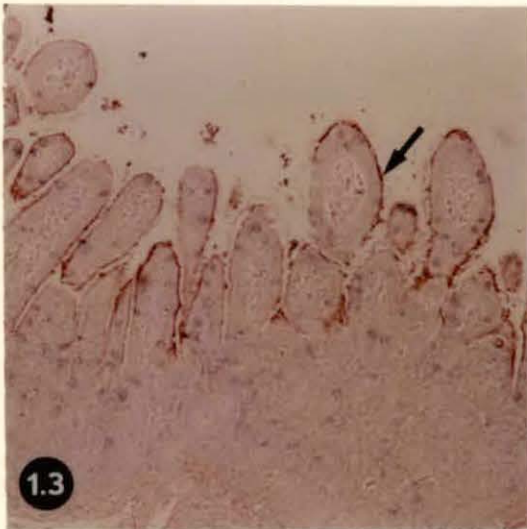
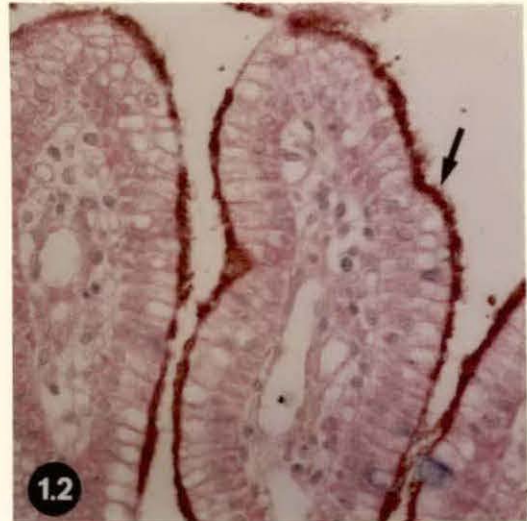
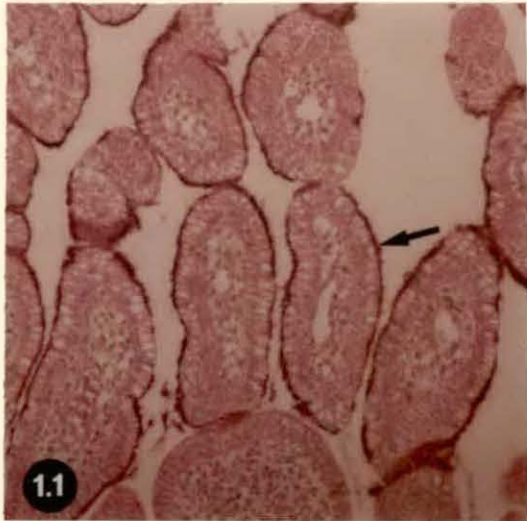


Table 1.1. Specificity of the PAP technique for K99 pilus antigen
in tissue

Tests	Blocking reagent	Tissue antigen	Primary antiserum (rabbit)	PAP staining
Tissue controls		K99	K99	+
		987P	K99	-
		Normal (none)	K99	-
Serum controls		K99	K99	+
		K99	K88	-
		K99	NRS ^a	-
Blocking controls	NRS	K99	K99	+
	Swine colostrum (K99 antibody)	K99	K99	-

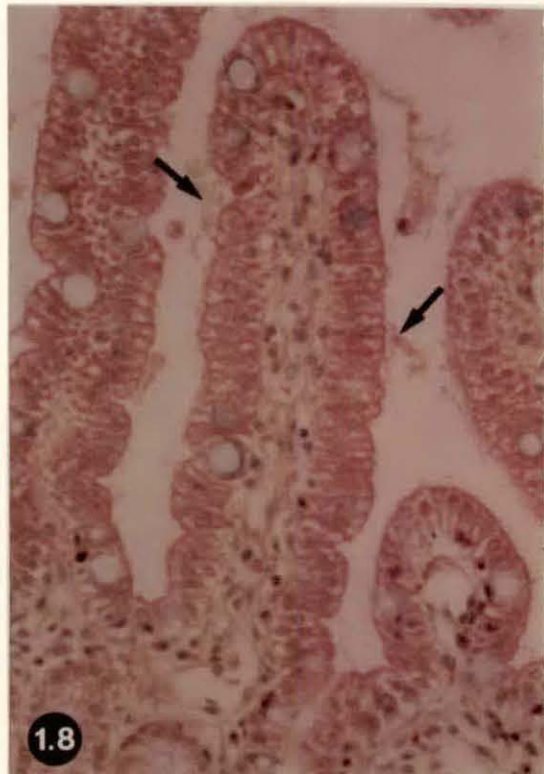
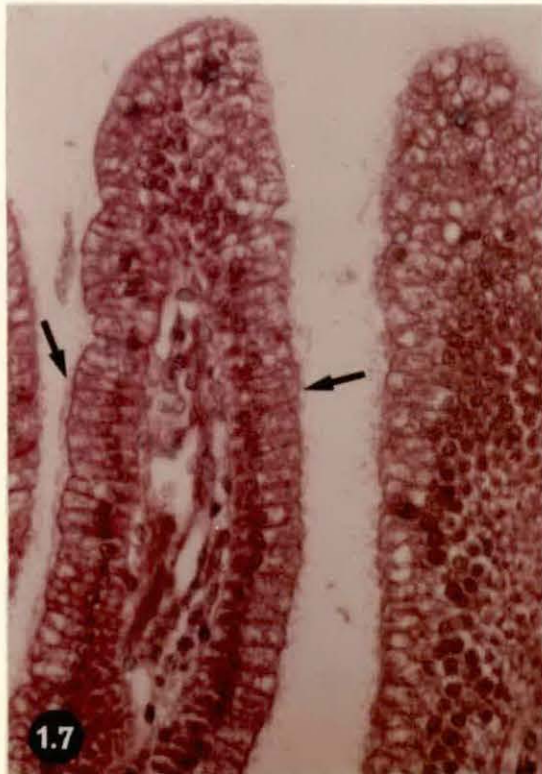
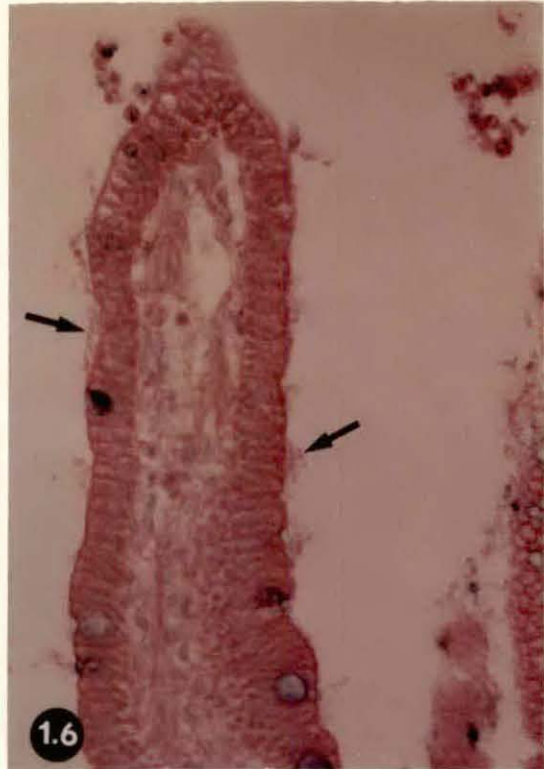
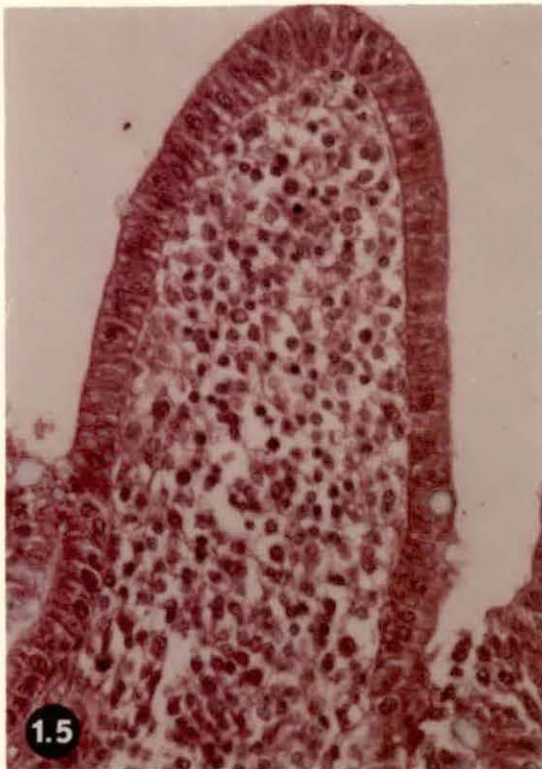
^aNormal rabbit serum.

Figure 1.5. Normal piglet ileum is not stained by PAP technique. Rabbit K99 antiserum employed in staining procedure. Ileum processed in cold methanol. Well-differentiated Giemsa stain

Figure 1.6. ETEC strain 987 colonizing villi of piglet ileum is not stained by PAP technique (arrows). Rabbit K99 antiserum employed in staining procedure. Ileum processed in cold methanol. Well-differentiated Giemsa stain

Figure 1.7. Normal rabbit serum employed in PAP staining procedure. ETEC strain 431 colonizing villi of piglet ileum is not stained (arrows). Ileum processed in cold methanol. Well-differentiated Giemsa stain

Figure 1.8. Rabbit K88 antiserum employed in PAP staining procedure. ETEC strain 431 colonizing villi of piglet ileum is not stained (arrows). Ileum processed in cold methanol. Well-differentiated Giemsa stain



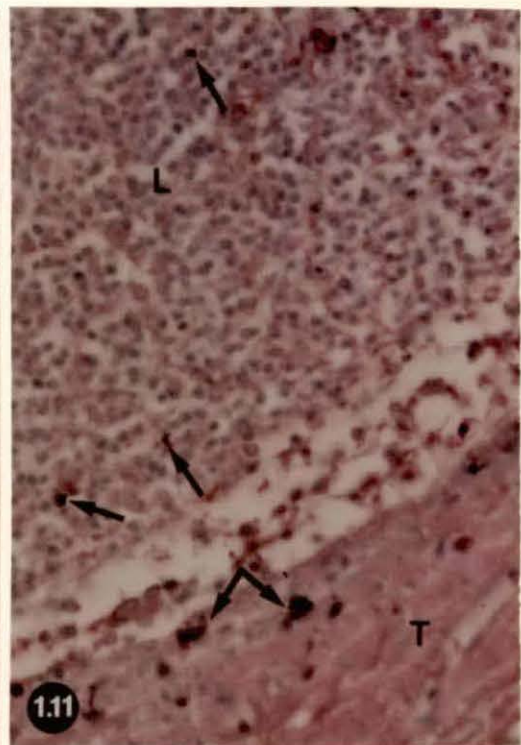
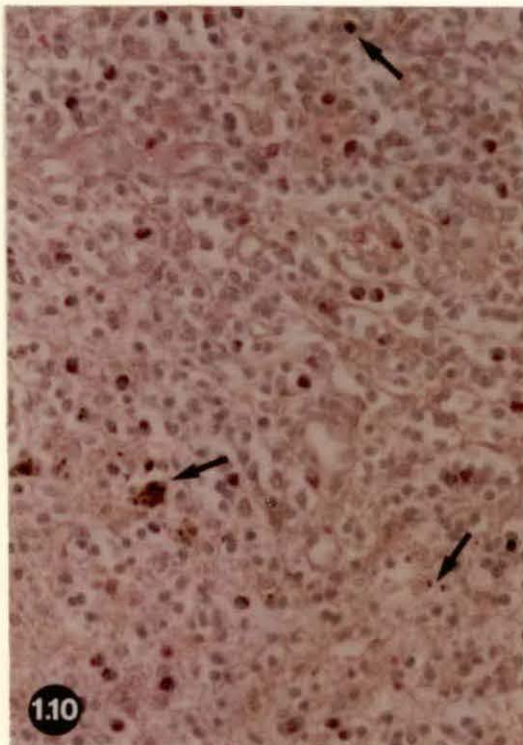
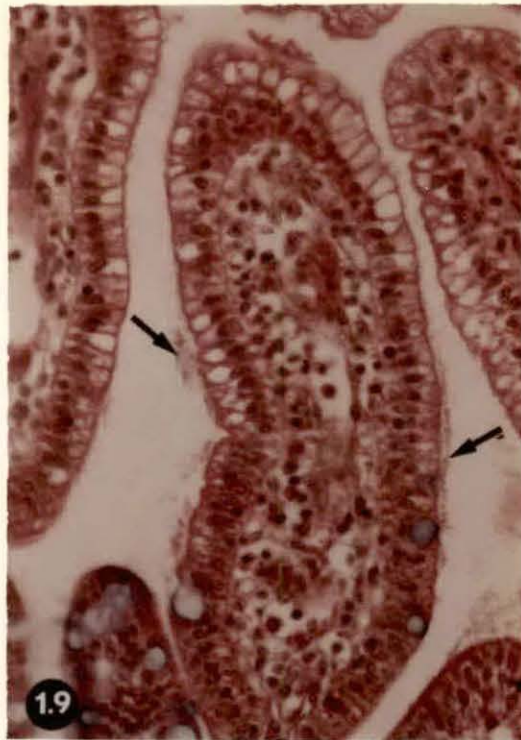
(Figures 1.7 and 1.8). Occasionally, diffuse background staining of tissue sections was encountered when NRS was employed as primary serum. ETEC strain 431 was not stained in this case, however. Applying swine colostrum with K99 antibody to sections prior to PAP staining effectively blocked positive staining of tissue containing K99 pilus antigen (Figure 1.9).

The ability of the PAP technique to detect a minute quantity of K99 pilus antigen in lymphoid tissue from an orally vaccinated gilt was difficult to evaluate. Sensitivity of the technique was evaluated with the assumption that ETEC strain 1459 expresses K99 pilus antigen in lymphoid tissue and that this antigen was present in the tissue sections observed. Positive staining granules were noted in tissue sections after rabbit K99 antiserum was employed as primary serum in the staining procedure. However, these granules were also present in tissue sections after NRS and rabbit K88 antiserum were substituted as primary sera in the staining procedure and after attempts were made to block the staining reaction with swine colostrum (K99 antibody). Further investigation revealed that treating gilt lymphoid tissue with absolute methanol containing 1% sodium nitroferricyanide and 1% glacial acetic acid prior to PAP staining could not completely eliminate endogenous peroxidase activity (Figure 1.10). Some, if not all, of these positive staining granules were then suspected to be endogenous peroxidase granules. The presence of these granules made localization of K99 pilus antigen, if present, very difficult when rabbit K99 antiserum was employed as primary serum in the staining procedure. Occasional superficial staining of tissue sections (artifact) and staining of lymph node trabeculae

Figure 1.9. Application of swine colostrum (K99 antibody) to piglet ileum prior to PAP staining. ETEC strain 431 colonizing villi is not stained (arrows). Ileum processed in cold methanol. Well-differentiated Giemsa stain

Figure 1.10. Residual peroxidase activity in lymph node of gilt (arrows). Lymph node processed in cold methanol and treated with absolute methanol containing 1% sodium nitroferricyanide and 1% glacial acetic acid. PAP technique was not performed. Well-differentiated Giemsa stain

Figure 1.11. Nonspecific staining (arrows) of gilt lymph node trabeculum (T). Superficial accumulation of reaction product, or artifact (arrows), on lymphoid tissue (L). Swine colostrum (K99 antibody) employed as blocking antiserum prior to PAP staining. Lymph node processed in cold methanol. Well-differentiated Giemsa stain



(nonspecific staining) was observed after swine colostrum (K99 antibody) was employed as blocking antiserum (Figure 1.11) and also after NRS was substituted as primary serum in the staining procedure. Nonspecific staining and the presence of artifact made localization of K99 pilus antigen, if present, even more difficult when rabbit K99 antiserum was employed as primary serum in the staining procedure.

DISCUSSION

Early in this study, it became apparent that antigenic reactivity of K99 pilus antigen could not be preserved in tissues routinely fixed in formalin and dehydrated in graded ethanols. Digestion of formalin-fixed tissue sections with trypsin did not appreciably enhance PAP staining. Antigenic reactivity was not preserved when tissues were processed in cold ethanol according to the method of Sainte-Marie (146). However, antigen localization and identification were impressive in tissues processed in cold methanol. The exact mechanism by which different alcohols denature proteins is not known (130). The decreased staining encountered when tissues were fixed in cold formalin, but dehydrated in cold methanol, was presumably due to the masking effect of formalin. The results of this study indicate that both fixation and dehydration may alter antigenic reactivity in tissues. In addition, temperature is another variable that must be considered when processing tissues.

Proper dilutions of the respective sera employed in the PAP technique are important in reducing background and nonspecific staining. A high dilution of the first antibody minimizes nonspecific attachment and guarantees reaction with K99 pilus antigen via both binding sites. An excess of the second antibody guarantees availability of a second binding site for reaction with the immunoglobulin contained in the PAP complexes. In this study, occasional nonspecific staining was encountered even though optimal dilutions of sera were employed.

Because of the biochemical nature of immunoenzyme techniques, buffer pH is very important and should be checked for accuracy before every

staining procedure. Water impurities and temperature variables that may have significant effects on Tris buffers must be kept at a minimum. Decreased staining intensity and increased solubility of reaction product in dehydrating alcohols are generally indicative of altered pH.

Diaminobenzidine that remains undissolved in the buffer can adhere nonspecifically to tissue sections, interfering with the interpretation of staining reactions. For this reason, the staining solution should be filtered as described. Other debris present in buffer and sera should be filtered out for the same reasons.

The presence of endogenous peroxidase activity in tissues can interfere with interpretation of the PAP reaction. The amount of endogenous peroxidase varies from one tissue to another; however, more of it is observed in lymphoid tissues. Absolute methanol containing 1% sodium nitroferricyanide and 1% glacial acetic acid destroyed endogenous peroxidase activity in piglet ileum. This treatment was superior to other methods described in the literature (80, 163). However, endogenous peroxidase activity was much stronger in gilt lymphoid tissue and could not be completely eliminated.

The PAP technique is a time-consuming procedure and experience is necessary to overcome technical problems often encountered. However, results are impressive in tissues where K99 pilus antigen is abundant. The special processing of tissues required in this procedure makes its use as a routine diagnostic tool doubtful.

CONCLUSIONS

The PAP technique as employed in this study was specific for ETEC K99 pilus antigen in paraffin-embedded tissue. Antigenic reactivity is optimal when tissues are processed in cold methanol according to a modified method of Sainte-Marie. This technique is applicable to tissues where antigen is abundant and where endogenous peroxidase activity is minimal. In this study, residual peroxidase activity, occasional nonspecific staining and the presence of staining artifact made interpretation of the PAP staining reaction very difficult when applied to gilt lymphoid tissue. Assuming that ETEC strain 1459 expresses K99 pilus antigen in gilt lymphoid tissue and that this antigen was present in the tissue sections evaluated, then the PAP technique does not appear to be sensitive enough to distinguish between K99 pilus antigen, residual peroxidase activity, and at times, nonspecific staining and artifact. Because of these limitations with the PAP technique, it was decided to conduct bacterial culture and agglutination studies to determine the distribution and persistence of two live K99 vaccines in selected tissues of orally vaccinated pregnant swine.

PART II: TISSUE DISTRIBUTION OF ENTEROTOXIGENIC ESCHERICHIA COLI
K99 PILUS ANTIGEN IN PREGNANT SWINE FOLLOWING FEEDING
OF A LIVE ORAL VACCINE

INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) in the small intestine of piglets produce a noninvasive infection that is characterized by profuse secretory diarrhea. The net result is dehydration, acidosis due to the loss of electrolytes, and death.

ETEC elaborate two types of enterotoxin which are capable of stimulating intestinal secretion. Heat-labile toxin or LT, is a high molecular-weight, immunogenic compound which loses its biologic activity after heating to 100°C for 15 minutes (34, 45, 64, 91). Heat-stable toxin or ST, is a small molecular weight, nonimmunogenic compound which does not lose its biologic activity after heating to 100°C for 15 minutes (2, 81, 157). Two forms of ST produced by swine ETEC have been described (24, 63, 120). Burgess et al. demonstrated that STa is methanol soluble, active in infant mice and ligated intestinal loops of piglets (1 to 3 days old), but inactive in weaned pigs (7 to 9 weeks old). STb is methanol insoluble, active in ligated intestinal loops of weaned pigs and rabbits, but inactive in infant mice (24). Recent investigation of ST suggests that its secretory effects may involve cyclic GMP (cGMP) (71). LT is believed to act via stimulation of intestinal adenylyl cyclase with resultant elevation of cyclic AMP (cAMP) levels (58). Elevated levels of cAMP prevent absorption of water and sodium ions by the enterocyte and stimulate active secretion of chloride ions (157). Enterotoxins are coded for by plasmids (65). Some ETEC produce both ST and LT (62, 151).

Prior to enterotoxin production, orally ingested ETEC adhere to and colonize absorptive cells of the intestinal mucosa. Three bacterial,

surface-associated pili (fimbriae) that mediate attachment have been studied in swine. These proteinaceous, antigenically distinct pili are designated K88, K99, and 987P. In piglets, the localization of ETEC depends on the type of pili associated with the respective strains of ETEC. Strains possessing the K88 pilus adhere to the mucosa throughout the small intestine. Strains possessing the 987P pilus adhere to the mucosa of the posterior small intestine, and strains possessing the K99 pilus adhere to the mucosa of the middle and posterior small intestine (152). Recent reports suggest that some ETEC produce more than one type of pilus (147). Evidence from in vitro adhesion assays support the notion that pilus receptors exist on enterocytes and that these receptors recognize only a single pilus type (77). Production of K88 and K99 pili is plasmid mediated (122. 123).

Colonization of the small intestine by ETEC stimulates a vigorous immune response principally involving immunoglobulin A (IgA). Furthermore, intestinal exposure to antigen often leads to the appearance of specific antibodies in the colostrum and milk of lactating swine. Porter et al. reported a preponderance of IgA- and IgM-producing cells in the intestinal lamina propria of young pigs orally inoculated with live and killed ETEC, with IgA predominating in the intestinal secretions (140). Kohler et al. orally vaccinated pregnant swine in late gestation with live ETEC for 3 consecutive days and demonstrated protection via colostrum for suckling piglets which were challenge inoculated with the homologous strain. Impaired colonization of the ileum by ETEC was noted histologically in protected piglets (88). Evans et al. demonstrated a significant increase in IgA in intestinal secretions and milk from sows

orally vaccinated with live ETEC. In addition, the immune response in the intestine was not as prolonged as that in the milk. Local intestinal antibody was present only in those sows continually fed live ETEC (49). Newby et al. fed live ETEC to weaned pigs and found that colonization of the intestine was required to induce a significant local IgA response (115).

In contrast, that response provoked by feeding killed ETEC is less marked. Stokes et al. found that large doses of killed ETEC were required to induce an intestinal IgA response in mice (161). Evans et al. fed killed ETEC to sows and demonstrated a delayed and reduced IgA response in the milk. IgM was the principal immunoglobulin detected in the milk of one sow, and prolonged feeding of large numbers of bacteria was required to elicit a response (49). However, Porter et al. reported that piglets continually fed large numbers of killed ETEC were less susceptible to challenge with live organisms at weaning (139). Chidlow and Porter found that prolonged feeding of killed ETEC, followed by parenteral vaccination with the somatic antigen, produced primarily IgM specific antibody in the colostrum of sows. They suggested that antigenic priming occurs in the intestine, and parenteral vaccination stimulates circulating, immunologically committed cells that originate in the intestine following oral immunization (31).

Moon demonstrated that pilus antigens are an important protective component of ETEC when given in oral vaccines. Gilts orally vaccinated with live ETEC produced colostrum antibody specific for the pilus antigens of the vaccine strains. Piglets suckling vaccinated gilts were resistant to challenge inoculation. In addition, the live oral ETEC

vaccines were much more effective in provoking an immune response than were killed ETEC and live rough non-ETEC (106).

The response to other intestinal pathogens has been documented. Bohl et al. demonstrated that sows naturally or experimentally infected with transmissible gastroenteritis (TGE) virus conveyed a high degree of passive immunity via IgA to their suckling piglets (13). Early studies with Vibrio cholerae demonstrated that guinea pigs orally inoculated with live organisms were immune to subsequent reinfection (27). More recently, Pierce et al. have shown that cholera toxin is a potent oral immunogen and affords protection against challenge with live organisms (136). The antigenic B subunit of cholera toxin lacks toxin activity, but avidly binds GM₁ ganglioside receptors present in most mammalian cell membranes (69, 133). This ability of the toxin to bind receptors at intestinal sites of immunopotentiality may explain its effectiveness as an immunogen (133). Like the ETEC enterotoxin LT, cholera toxin activates membrane-bound adenylyl cyclase, which results in elevated intracellular levels of cAMP (53). Lymphocytes have been shown to respond to elevated intracellular levels of cAMP by increasing antibody synthesis in the presence of antigen (78, 79).

Live oral ETEC vaccines have been used successfully in veterinary medicine (87). However, the nature of the immune response and the mechanism of antigen processing after oral administration of ETEC remains unclear. In this regard, emphasis has been placed on the role of the intestinal Peyer's patches as a preferential site of antigen uptake, and as a significant contributor to the development of intestinal and mammary secretory IgA responses.

Peyer's patches (PP) are multiple, confluent aggregates of lymphoid tissue in mammalian intestine and are a major component of gut-associated lymphoid tissue (GALT). These aggregates are found in the antimesenteric wall of the intestine. In swine, PP are band-like and extend from the anterior duodenum to proximal regions of the cecum and colon (150). Their distribution becomes more diffuse and their numbers increase in distal portions of the intestinal tract (32). Solitary lymphoid nodules (SLN) are the major component of GALT in the colon (150).

Histologically, PP are nodular aggregates of lymphoid tissue in the lamina propria. They generally extend into the submucosa, disrupt the muscularis mucosae and spread out into loose connective tissue of the submucosa. Each nodule is surrounded by efferent lymphatics which drain predominately to the mesenteric lymph nodes, with vessels occasionally extending into the immediate lamina propria (129). There are no afferent lymphatics from the lamina propria to the PP. Each nodule consists of a germinal center, or B-cell region, and an interfollicular area, or T-cell region. That portion of the nodule that projects into the intestinal lumen is referred to as the dome. Circulating T and B lymphocytes enter PP through modified postcapillary venules in the interfollicular regions and subepithelial areas of the domes (137).

Evidence that PP respond to the presence of antigen in the intestine has been documented. Pollard and Sharon reported that PP in germ-free mice do not develop to maturity (137). Perey and Good demonstrated that exposure to bacterial flora was essential for full development of rabbit PP and humoral immunity (131). Kenworthy noted that development of GALT in germ-free piglets was retarded. However, normal development proceeded

after oral inoculation with E. coli (85). Müller-Schoop and Good demonstrated an increased responsiveness of PP lymphocytes to orally administered BCG and to the presence of indigenous microflora (111). Keren et al. inoculated ligated ileal loops in rabbits with an invasive, hybrid strain of Shigella and reported that loops containing a PP exhibited an earlier and stronger IgA response as compared to loops lacking a PP. They suggested that local immunity achieved in loops without a PP may have resulted from local bacterial invasion and stimulation of immunocompetent cells in the lamina propria or mesenteric lymph nodes (MLN) (86).

Uptake of luminal antigen is facilitated by a specialized lympho-epithelium overlying the domes of PP. Faulk et al. observed PP in rabbits and described this dome epithelium to be noncolumnar and to contain clusters of lymphocytes (51). Owen and Jones described the presence of "M" (membranous) cells in the dome epithelium of humans. These cells were covered with irregular ridges instead of microvilli and were interspersed between microvillus-covered, noncolumnar cells. Lymphocytes were observed lying within a reticulum formed by the M cells. In some areas, lymphocytes lying within M cell processes were 0.3 μm from the intestinal lumen. The authors suggested that these lymphocytes may be positioned to respond to antigenic material within the M cells (125). Ultrastructural features of M cells include mitochondria, glycogen, Golgi apparatuses, and multiple cytoplasmic vesicles. Histochemical studies have demonstrated a preferential uptake of horseradish peroxidase by M cells after exposure of the intestine to small quantities of this antigen. After uptake by M cells, the enzyme was released into the

interstitial space and subsequently taken up by lymphocytes via pinocytosis (124). Chu orally inoculated 8-week-old pigs with TGE virus and detected viral particles in cytoplasmic vesicles of M cells. Viral particles were also noted in macrophages and lymphocytes in the dome (32). In another study, reovirus was shown to enter lymphoid follicles of mice via M cells (174). Owen and Nemanic observed the presence of an unknown species of bacterium between M cells and adjacent absorptive cells in nude mice. They then suggested that M cells were probably not susceptible to bacterial penetration (126). More recently, Owen et al. reported the uptake of Vibrio cholerae organisms by M cells in rabbit PP. Phagocytosed organisms were subsequently released into interstitial spaces and taken up by lymphocytes and macrophages (127). Cantey and Inman reported that the RDEC-1 strain of E. coli preferentially attached to and colonized the dome epithelium of PP in rabbits before colonization of ileum, cecum, and colon began (29). M cells were shown to be the initial, specific sites of attachment for this strain (75). Systemic spread of Listeria and Salmonella is initiated through PP (30, 67, 97). It is not known whether these bacteria adhere to or invade through M cells.

Most investigations have failed to detect the presence of locally produced antibody in PP following oral or parenteral immunization (11, 66). Faulk et al. could not detect IgA-bearing plasma cells or specific IgA synthesizing immunoblasts in PP of normal or hyperimmune animals (51). Craig and Cebra demonstrated that PP contain a precursor population of cells committed to IgA synthesis. Lethally irradiated rabbits were inoculated i.v. with donor lymphoid cells from various

sites. IgA-producing cells were observed in the intestine of recipient animals inoculated with PP cells, but not in the intestine of recipients inoculated with lymphoid cells from other sites (40). Other studies demonstrated that PP were the source of IgA-precursor cells homing to the lamina propria (41). Gowans and Knight reported that lymphocytes from MLN and the thoracic duct selectively localize in the intestine (55). In other studies, transfer of PP, MLN, and thoracic duct cells between syngeneic mice was examined. MLN and thoracic duct cells localized in the intestine and became IgA-producing plasma cells much sooner than did PP cells (60, 104). It was suggested that precursor cells from PP migrate to MLN and the thoracic duct via lymphatics and mature during this migration (60). McWilliams et al. demonstrated that MLN are the major source of IgA-producing plasma cells in the intestine. Precursor cells from PP become committed to IgA synthesis and develop surface IgA before emigrating from MLN, to the thoracic duct, and into circulation (105). Bennell and Husband have recently reported that recirculating lymphocytes leave MLN via blood capillaries rather than the efferent intestinal lymph in swine (6, 7). Gearhart and Cebra suggested that antigen uptake by M cells may be important in promoting proliferation of recirculating memory cells that have been primed outside of GALT (54).

The mechanism by which IgA-committed cells selectively localize in the intestinal mucosa is not known. Initially, it was postulated that these cells homed to sites where antigen was localized. Husband and Lascelles injected different antigens into two Thiry-Vella intestinal loops in sheep. An IgA response against each antigen appeared in the respective immunized loop and never in both loops (74). Identical

results were observed in rats in which Thiry-Vella loops were employed (72). Pierce and Gowans demonstrated the presence of specific IgA-producing cells in the intestine of rats orally inoculated with cholera toxoid. The density of cells was highest in regions of the intestine directly challenged with antigen (135). Husband and Gowans reported that IgA-committed cells can migrate to the intestinal mucosa independently of antigen, but antigen has a profound effect on the location, magnitude, and persistence of the response (73). Brandtzaeg suggested that secretory component (SC) on the surface of columnar epithelial cells serves as a receptor for migrating IgA-committed cells (15). Other studies indicate that SC is probably not directly involved in the homing process (104). Butcher et al. demonstrated that migrating lymphocytes may selectively interact with specialized endothelium in the postcapillary venules of lymphoid tissues (28). Whether this idea applies to migrating IgA-committed cells is not known (92).

Hormones have been shown to influence the homing of IgA-committed cells from PP to the mammary gland. Roux et al. demonstrated that IgA-committed cells from MLN of mice homed to the mammary gland of syngeneic recipients in late pregnancy and during lactation. Homing did not occur in virgin mice, in early pregnancy, or after weaning (145). Weisz-Carrington et al. reported a significant increase in IgA-secreting plasma cells in the mammary glands of virgin mice treated with estrogen, progesterone, and prolactin. They suggested that a hormone-induced increase in epithelial receptors during lactation might attract more IgA-committed cells (171). Kühn and Kraehenbuhl reported the presence of receptors for

polymeric IgA in rabbit mammary glandular epithelium. These receptors are transmembrane glycoproteins, structurally related to SC (90).

Much of the IgA found in intestinal and mammary secretions is synthesized and secreted locally by plasma cells (167). This IgA is synthesized as an 11S dimer and contains an additional polypeptide chain known as the joint (J) chain (89). The J chain is synthesized in the same cells which produce dimeric IgA (16). In contrast, serum IgA is a 7S monomer, with a small percentage existing in polymeric form. IgA dimers bind SC to form secretory IgA (SIgA) upon passage through crypt epithelium in the intestine and glandular epithelium of the mammary gland. SC is synthesized by the epithelial cells (3, 18, 90). At least seven models have been postulated to explain translocation of dimeric IgA across epithelial cells (19). A more current model proposed by Brandtzeag suggests that dimeric IgA binds SC at the basolateral portion of the epithelial cell membrane. These dimer IgA-SC complexes are then taken into the cell via pinocytosis and released into the lumen via exocytosis. Or, these complexes may partially float in the plasma membrane and be released into the lumen without entering the cytoplasm. The preferred mechanism would depend on the cellular distribution of SC (20). SIgA has also been shown to enter the intestinal lumen via the bile. Recent studies have demonstrated that dimeric IgA synthesized at one mucosal site is selectively transported across hepatocytes into the bile (52, 95, 121). Transport is dependent on the presence of SC on the surface of hepatocytes (52, 155).

The significance of SIgA is not clear. SIgA does not promote phagocytosis (173, 176), but it does possess strong agglutinating

activity (102). Although it does not fix complement, SIgA has been shown to lyse E. coli in the presence of complement and lysozyme (1). SIgA is not absorbed from the intestine of neonate piglets (138), and is quite resistant to digestion by proteolytic enzymes (96, 149). Walker and Isselbacher suggest an "immune exclusion" role for secretory antibodies in the intestine. These antibodies prevent bacterial adherence, neutralize viruses, and counteract enterotoxins. Formation of antigen-antibody complexes on the surface of the intestine facilitates the removal of harmful substances by other nonimmunologic protective mechanisms (170).

The finding of large amounts of IgM in secretions and plasma cells in mucosal tissues of IgA-deficient humans suggests that IgM is also produced locally (21). IgM exists primarily as a 19S pentamer, contains the J chain (89), and is capable of binding SC (17). Cooper and Turner suggested that PP act as a source of IgM memory cells which migrate to other lymphoid tissues (37). MLN were shown to be a major source of IgM precursor cells migrating to the intestine (103). It has been suggested that cells bearing IgM evolve to produce IgG and then IgA (38); however, more recent evidence does not support this hypothesis (82). Allen and Porter reported that PP of unweaned piglets contained a majority of IgM precursor cells. Because both IgA and IgM immunoglobulins were demonstrated in crypt epithelium of these piglets, they suggested that these two immunoglobulins may have complementary roles as intestinal antibodies (4). An IgM response appears early in piglets orally inoculated with live and killed ETEC (140). Specific IgM antibody has been demonstrated in milk of sows orally vaccinated with live and killed ETEC (49) which reinforces the notion of an existing enteromammaric link. IgM is

susceptible to digestion by proteolytic enzymes (143) which tends to suggest a minor role for this immunoglobulin in the intestine.

Whether macrophages are capable of processing antigen and presenting it to immunocompetent lymphocytes in PP is not clear (175). Kagnoff and Campbell reported that mouse PP contained antigen-sensitive T and B lymphocytes, but lacked the macrophage-like accessory adherent cells necessary for a primary humoral response and cell-mediated cytotoxicity in vitro (83). More recently, Richman et al. demonstrated that collagenase-treated, macrophage-enriched cells from PP of mice were capable of presenting antigen to primed T cells. The role of collagenase in this respect was unknown (144). Macrophages have been demonstrated in the thoracic duct following mesenteric lymphadenectomy (98). Bienenstock et al. suggested that macrophages may have a selective migration pattern and traffic between mucosal surfaces (12).

The function of intraepithelial lymphocytes is unclear. These cells were shown to be T cells which selectively home from MLN and the thoracic duct to intestinal lamina propria and villous epithelium (60). There is evidence that MLN T blasts do not localize in the mammary gland (99). Some authors have suggested that these cells are primed T lymphocytes which have returned to the intestine after exposure to antigen in PP and are a manifestation of cell-mediated response to this antigen (61).

Lymphoepithelium overlying SLN has been shown to contain M cells (165). These nodules, distributed throughout the intestine, are thought to function in a manner analogous to PP.

The role that tonsils assume in secretory immunity is unclear. Epithelial cells overlying tonsillar crypts are phagocytic and thought to

process antigen (172). Histochemical studies have shown that IgA is not the predominant tonsillar immunoglobulin, and SC is not present at this site. It has been suggested that tonsils are immunologically unrelated to other components of GALT (44).

Investigations of some laboratory animal systems indicate that the oral immunogenicity of some bacterial strains depends on their ability to survive and multiply within PP (68). Moon has suggested that the ability of live ETEC to elicit a secretory immune response in swine may reside in their ability to multiply and persist in PP. Killed ETEC and live rough non-ETEC would be ineffective in this respect (106).

STATEMENT OF PROBLEM AND OBJECTIVES

The role of gastrointestinal mucosal surfaces and their associated lymphoid components in swine orally vaccinated with ETEC remains largely uninvestigated. Determining the distribution of both live ETEC and a weakly immunogenic strain of non-ETEC would enhance the understanding of secretory immunity in this species. Perhaps then, attempts at delivering sufficient killed or attenuated antigen to sites critical for a secretory immune response might be attempted.

This study will be undertaken to determine the distribution and persistence of two live K99 vaccines in selected tissues of orally vaccinated pregnant swine. Animals will be orally vaccinated with a strain of live wild type ETEC or a strain of live rough non-ETEC.

MATERIALS AND METHODS

Animals

Two nonpregnant gilts and two nonpregnant sows were employed in a pilot study. These animals had served as uninoculated controls in experimental studies at the National Animal Disease Center (NADC), Ames, Iowa. In addition, sixteen pregnant gilts were obtained from a herd with no history of colibacillosis or of vaccination to prevent this disease. These animals were moved to NADC 1 month before their anticipated farrowing dates and were observed in isolation for 2 weeks. All animals were housed in isolation rooms while being fed their respective vaccines.

Vaccines

The live ETEC vaccine was the Troyer strain (09:K35:K99:NM). This strain has been shown to be an effective live oral ETEC K99 vaccine in swine (106). A live rough non-ETEC strain of K12 bearing K99 pilus antigen (153) was employed for comparative purposes. This strain has been shown to be an ineffective oral immunogen in swine (106). Both cultures were grown aerobically in broth (Trypticase soy broth-BBL, Becton, Dickinson, Cockeysville, MD) for 16 hours at 37°C on an oscillating shaker.

Vaccination

Pregnant gilts were fed vaccine 2 weeks before their anticipated farrowing dates. All animals were fasted for 24 hours and fed the Troyer or K12(K99⁺) strains by mixing broth culture with 1.4 kg of feed. Each animal was vaccinated by feeding 200 ml of fresh broth culture/day (10^{11}

E. coli/day). Animals killed later than 24 hours' post-vaccination were maintained on 1.4 kg of feed/day until necropsy. Animals fed vaccine more than one time were examined for diarrhea 1 day after each feeding. Vaccination schedules are given in Tables 2.1, 2.2 and 2.3.

Serum

Serum samples were collected from all pregnant gilts upon arrival at NADC and again at necropsy. Titers of K99 antibody were determined by enzyme-linked immunosorbent assay (ELISA) (48). The K99 antigen used in the assay was prepared (76) from a K12 strain of E. coli carrying the K99 pilus. Standard K99 antiserum was prepared against E. coli strain K12(K99⁺), absorbed (108), conjugated to alkaline phosphatase, and used as the indicator antiserum. Serum was not collected from nonpregnant animals used in the pilot study.

Necropsy, specimen collection, and processing

Animals were killed with an intramuscular injection of 10% succinylcholine chloride solution followed by exsanguination. The esophagus and rectum were ligated and then incised. Viscera was removed using a disinfected necropsy knife and sterile surgeon's gloves. Tissue specimens were collected on a disinfected necropsy table. Lymphoid tissues collected for bacterial culture included: tonsil, medial retropharyngeal lymph node draining the tonsil, gastric and mesenteric lymph nodes (jejunal, midsmall intestinal, ileocolic), and ileal Peyer's patch. Mucosal tissues collected for bacterial culture included: stomach (fundus), jejunum, midsmall intestine, mesenteric border of the ileum, cecum, and apex of the spiral colon. Ileal tissues were taken

approximately 1 m cranial to the ileocecal junction. Jejunal tissues were taken approximately 1 m caudal to the ligament of Treitz. Midsmall intestine was collected halfway between the ileal and jejunal segments. All tissues were aseptically collected using sterile forceps and scissors for each specimen. Lymph nodes were collected first followed by ileal Peyer's patch and mucosal surfaces. Ingesta that adhered to mucosal surfaces was included in the culture studies. Feces were collected from the rectums of pregnant animals. All culture specimens were placed in individual plastic bags and frozen at -70°C within 20 minutes of collection. Ileal Peyer's patch was collected from pregnant gilts for histology. This tissue was fixed in 10% neutral phosphate buffered formalin, placed in tissues cassettes, dehydrated in graded ethanols, and cleared in xylene. Tissues were infiltrated with paraffin (Paraplast, Lancer, St. Louis, MO) using an autotechnicon (Autotechnicon® Mod. 2A, Technicon, Tarrytown, NY) and embedded. Five micron sections were cut and stained with hematoxylin and eosin (H & E) and by the New Gram method (22). Six sections of one ileal Peyer's patch were collected from each pregnant gilt for electron microscopy. One millimeter-thick sections were fixed in 2.5% glutaraldehyde for 3 hours, rinsed twice in cacodylate buffer, pH 7.4, post-fixed in 1% osmium tetroxide for 2 hours, dehydrated in graded ethanols, cleared in propylene oxide, and polymerized in epoxy resin (Epon 812, Ted Pella, Tustin, CA). One micron-thick sections were cut and stained with alkaline toluidine blue (1% toluidine blue in 1% sodium borate) and examined by light microscopy. Ultrathin sections were cut with an LKB 8800 ultramicrotome (LKB-produkter AB, LKB Instruments, Inc., Rockville, MD), stained with uranyl acetate and lead citrate, and

examined with a Hitachi H-500 transmission electron microscope (Hitachi, Mountain View, CA) operated at 75 KV.

Bacterial culture

Tissue samples were allowed to thaw. Using sterile instruments, approximately 1 gram of tissue was added to 30 ml of 0.3% peptone (Bacto-Peptone, Difco, Detroit, MI) water. The exact weight of each tissue was recorded and the tissues were homogenized (Virtis 45 homogenizer, The Virtis Co., Gardiner, NY). A Spiral Plater (Spiral Plater Mod. C, Spiral Systems, Inc., Cincinnati, OH) was used to inoculate 0.1 ml of the homogenate onto agar plates. Tissue homogenates from animals vaccinated with the Troyer strain were inoculated onto Minca agar plus IsoVitale X (Minca IS, BBL Microbiology Systems, Cockeysville, MD) (59) containing 256 µg streptomycin/ml and 32 µg ampicillin/ml (107). These plates were incubated aerobically for 24 hours at 37°C. The number of colony-forming units (CFU) resembling Troyer strain was recorded. Three translucent (acapsular bacteria) colonies were then transferred to Minca IS agar without dextrose (59) and without antibiotics. These plates were incubated aerobically for 72 hours at 37°C. Bacterial colonies were then tested for agglutination by K99 antiserum. Agglutinable colonies were considered to be the Troyer strain and bacterial counts were expressed as \log_{10} CFU/gm tissue. Tissue homogenates from animals vaccinated with the K12(K99⁺) strain were inoculated onto MacConkey agar containing 50 µg nalidixic acid/ml (50) and then incubated aerobically for 48 hours at 37°C. The number of CFU resembling the K12(K99⁺) strain was recorded. A few colonies were then transferred to tryptone (Bacto-Tryptone, Difco)

broth agar, incubated aerobically for 48 hours at 37°C, and then tested for agglutination in K99 antiserum. Agglutinable colonies were considered to be the K12(K99⁺) strain and bacterial counts were expressed as log₁₀ CFU/gm tissue. Failure to culture either vaccinal strain from tissue specimens after two attempts was recorded as N (not isolated; log₁₀CFU/gm tissue < 2.5).

Agglutination

Rabbit K99 antiserum (absorbed standard 1:10)(108) was provided by the Colibacillosis Research Unit of NADC. This serum was considered to be monospecific K99 pilus antiserum. Bacterial colonies grown on Minca IS agar without dextrose and without antibiotics (Troyer strain) or tryptone broth agar [K12(K99⁺) strain] were tested for agglutination by the slide agglutination method (156). Monospecific K99 antiserum and normal rabbit serum (NRS) were diluted 1:10 in physiological saline containing 0.5% phenol and were used as the test sera. Bacterial growth that demonstrated grossly apparent agglutination in K99 antiserum and were not agglutinated in NRS were recorded as K99 positive.

RESULTS

All animals consumed their respective vaccines. None of the animals developed diarrhea during the experiment. Pregnant gilts were sero-negative for K99 antibody before vaccination and again at necropsy (serum not taken in pilot study).

Early attempts to recover the Troyer strain from gilts 1 and 2 in the pilot study were unsuccessful (Table 2.1). This strain was recovered from the gastrointestinal tract and most lymphoid tissues of sow 2 but was not recovered from the gastrointestinal tract and associated lymphoid tissues of sow 1. It was hypothesized that feeding vaccine for 3 days may have boosted immunity such that the distribution, number, or persistence of the Troyer strain was reduced.

Most pregnant gilts vaccinated with the Troyer strain for 3 successive days did not show evidence of an existing booster effect (Table 2.2). Distribution of this strain along the intestine was similar in most gilts killed 6 hours after vaccination, regardless of feeding duration. The Troyer strain was not isolated from the small intestine (except Peyer's patch) of one 6-hour gilt (#2184) vaccinated on 3 successive days. Isolation of this strain from the stomach and lymphoid tissues varied among 6-hour gilts; however, relatively high numbers of bacteria were consistently recovered from the ceca, colons, and feces of these animals. The numbers of Troyer strain diminished in the small intestine between 6 and 24 hours' post-vaccination. This strain was recovered from the ceca, colons, and feces of 24-hour gilts, however. Low numbers of the Troyer strain were isolated from Peyer's patches, but

Table 2.1. Pilot study. Tissue distribution of the Troyer strain in orally vaccinated nonpregnant animals (\log_{10} CFU/gm tissue)

	Gilt 1 ^a	Gilt 2 ^a	Sow 1 ^b	Sow 2 ^c
Duration of vaccine feeding (days)	3	3	3	1
Interval between final vaccine feeding and necropsy	24 hrs	7 days	6 hrs	6 hrs
Stomach	N ^d	N	N	4.7
Jejunum	N	N	N	4.4
Midsmall intestine	N	N	N	3.9
Ileum, Peyer's patch	N	N	N	4.0
Ileum, mesenteric border	N	N	N	3.4
Cecum	N	N	N	6.2
Colon	N	N	N	6.3
Tonsil	N	N	- ^e	- ^e
Retropharyngeal ln ^f	N	N	2.9	2.5
Gastric ln	N	N	N	N
Jejunal ln	N	N	N	3.1
Midsmall intestinal ln	N	N	N	3.3
Ileocolic ln	N	N	N	3.5

^aFirst attempt to recover vaccinal strain.

^bSecond attempt to recover vaccinal strain.

^cThird attempt to recover vaccinal strain.

^dVaccinal strain not isolated (\log_{10} CFU/gm tissue < 2.5).

^eFungal contamination.

^fLymph node.

Table 2.2. Tissue distribution of the Troyer strain in orally vaccinated pregnant gilts (\log_{10} CFU/gm tissue)

Gilt No.	603	657	2080	2086	2078	2184	495	578	590	579
Duration of vaccine feeding (days)	1	1	1	3	3	3	1	1	1	1
Interval between final vaccine feeding and necropsy (hrs)	6	6	6	6	6	6	24	24	48	48
Stomach	N ^a	4.3	2.5	N	2.5	5.3	N	N	N	N
Jejunum	3.1	4.1	2.7	4.2	2.3	N	N	N	N	N
Midsmall intestine	4.9	4.3	2.9	5.0	2.8	N	N	N	N	N
Ileum, Peyer's patch	5.5	4.3	3.4	3.3	3.5	2.5	2.5	2.4	N	N
Ileum, mesenteric border	5.1	4.0	3.3	2.8	3.6	N	N	N	N	N
Cecum	6.2	6.4	8.0	6.5	6.7	4.9	3.9	5.0	N	N
Colon	6.5	6.8	8.1	6.4	6.8	5.3	4.2	4.1	N	N
Feces	7.2	5.2	8.0	7.1	7.7	5.9	5.2	5.3	N	N
Tonsil	3.2	4.4	2.9	N	3.9	N	N	N	N	N
Retropharyngeal ln ^b	N	N	N	N	3.9	N	N	N	N	N
Gastric ln	N	N	N	N	N	N	N	N	N	N
Jejunal ln	N	N	N	2.9	3.6	N	N	N	N	N
Midsmall intestinal ln	N	N	N	2.9	3.6	N	N	N	N	N
Ileocolic ln	N	N	N	3.0	3.3	N	N	N	N	N

^aVaccinal strain not isolated (\log_{10} CFU/gm tissue < 2.5).

^bLymph node.

not from lymph nodes of gilts killed 24 hours after vaccination. It could not be determined whether these bacteria were within Peyer's patches or on the surface. The Troyer strain was not recovered from gilts 48 hours after vaccination.

The K12 (K99⁺) strain was infrequently recovered from the small intestine and lymphoid tissues (tonsil) of pregnant gilts 6 hours after vaccination (Table 2.3). This strain persisted in the ceca, colons, and feces for 24 hours but could not be recovered 48 hours after vaccination.

Ileal Peyer's patches from pregnant gilts were easily identified by light microscopy (Figure 2.1). The dome epithelium was infiltrated by lymphocytes which appeared to distort absorptive cells (Figure 2.2). Occasionally a few goblet cells were present within the dome epithelium. Ten Gram-stained sections of Peyer's patch from each pregnant gilt were examined for the presence of bacteria. Gram-negative bacteria were observed in the domes in 6 of 6, 6-hour gilts vaccinated with the Troyer strain. These bacteria were noted in subepithelial areas and were often closely associated with, or present within, the dome epithelium (Figures 2.3 and 2.4). Gram-negative bacteria were also observed in the domes in 1 of 2, 24-hour gilts vaccinated with the Troyer strain and in 1 of 2, 6-hour gilts vaccinated with the K12 (K99⁺) strain. Bacteria were not detected in Peyer's patches of the other 8 pregnant gilts in this study. Neutrophils appeared to be especially prominent in the domes and on the dome epithelium of 6-hour gilts vaccinated with the Troyer strain.

Six sections of ileal Peyer's patch from each pregnant gilt were examined by electron microscopy. Macrophages containing phagocytosed

Table 2.3. Tissue distribution of the K12 (K99⁺) strain in orally vaccinated pregnant gilts (\log_{10} CFU/gm tissue)

Gilt No.	954	494	616	294	612	408
Duration of vaccine feeding (days)	1	1	1	1	1	1
Interval between final vaccine feeding and necropsy (hrs)	6	6	24	24	48	48
Stomach	N ^a	3.2	N	N	N	N
Jejunum	N	N	N	N	N	N
Midsmall intestine	N	N	N	N	N	N
Ileum, Peyer's patch	N	N	N	N	N	N
Ileum, mesenteric border	3.2	N	N	N	N	N
Cecum	3.5	4.2	N	3.3	N	N
Colon	4.6	3.3	3.2	3.1	N	N
Feces	5.1	3.3	3.2	3.4	N	N
Tonsil	N	3.3	N	N	N	N
Retropharyngeal ln ^b	N	N	N	N	N	N
Gastric ln	N	N	N	N	N	N
Jejunal ln	N	N	N	N	N	N
Midsmall intestinal ln	N	N	N	N	N	N
Ileocolic ln	N	N	N	N	N	N

^aVaccinal strain not isolated (\log_{10} CFU/gm tissue < 2.5).

^bLymph node.

Figure 2.1. Ileal Peyer's patch lymphoid follicle (F) and associated dome (D). H & E stain. Gilt 612

Figure 2.2. Dome epithelium (DE) infiltrated by lymphocytes. Note close proximity of lymphocytes to ileal lumen (arrows). H & E stain. Gilt 612

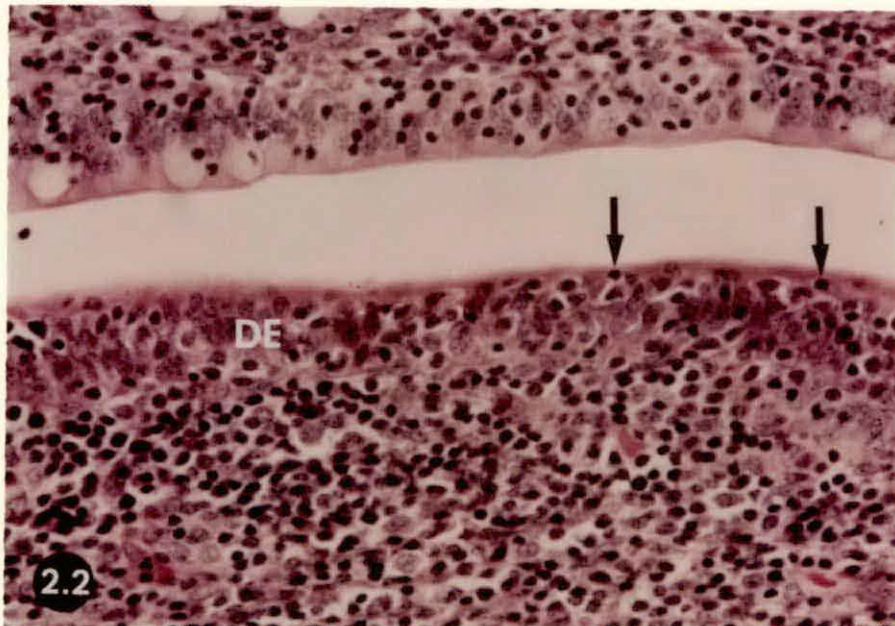
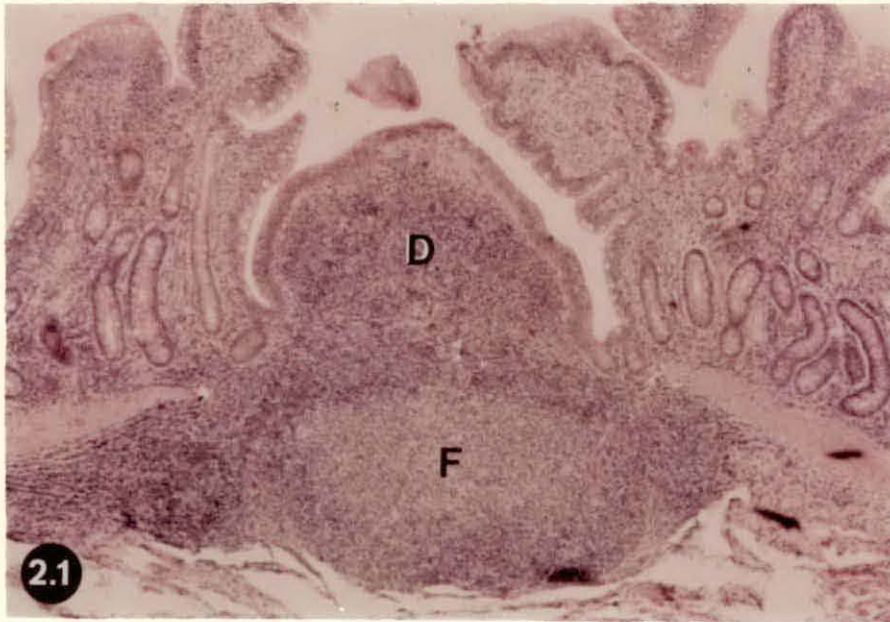
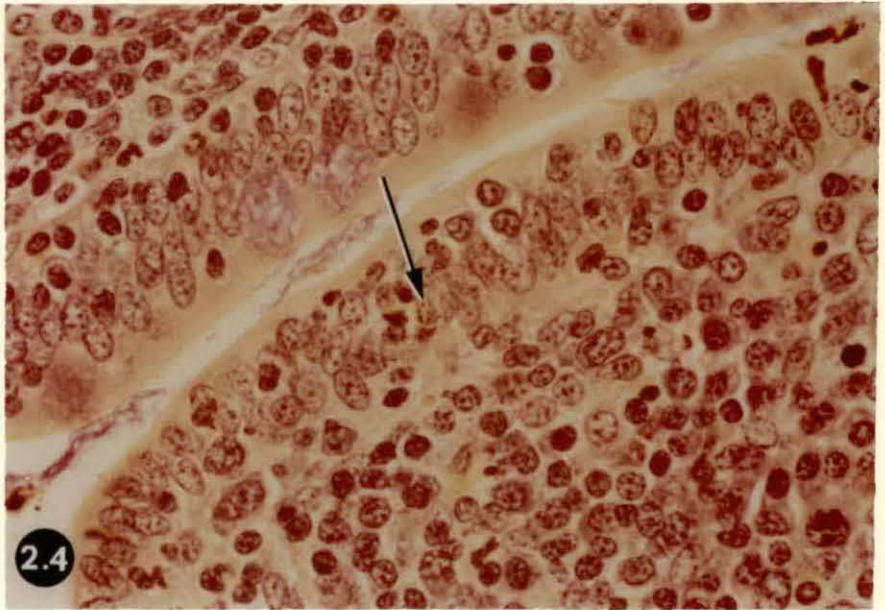
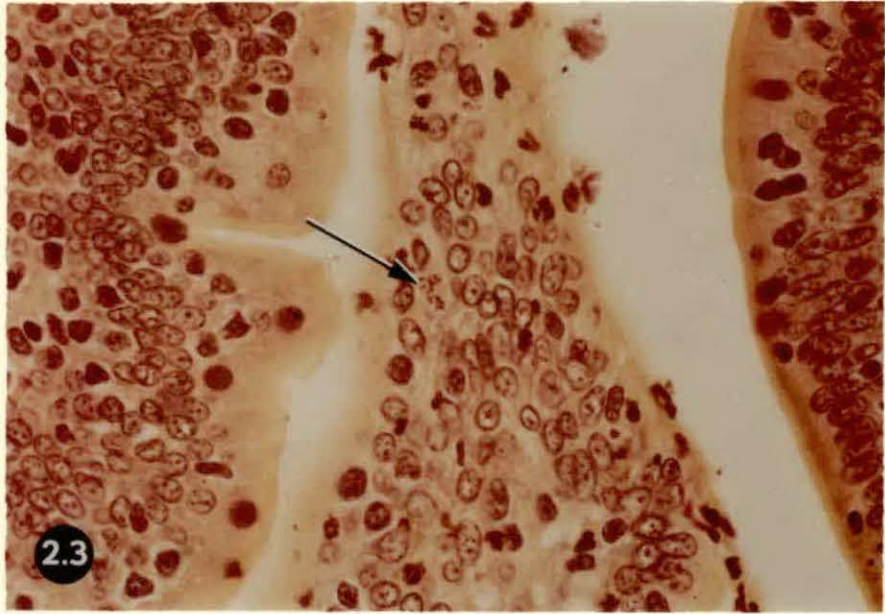


Figure 2.3. Gram-negative bacteria (arrow) in close association with the dome epithelium of 6-hour gilt orally vaccinated with the Troyer strain. New Gram stain. Gilt 2086

Figure 2.4. Gram-negative bacteria (arrow) present within the dome epithelium of 6-hour gilt orally vaccinated with the Troyer strain. New Gram stain. Gilt 603



bacteria were observed in subepithelial areas of the domes in 6 of 6, 6-hour gilts vaccinated with the Troyer strain. Many phagocytosed bacteria resembled E. coli. These bacteria generally measured 0.5 μ by 1.0 μ to 2.0 μ and were characterized by a slightly rugose cell wall (Figure 2.5). Occasionally, bacteria were associated with necrotic cells (Figure 2.6). Bacteria were not observed within or between absorptive cells of the dome epithelium. Cells resembling M cells were also devoid of bacteria. Bacteria were not detected in Peyer's patches of other gilts in this study. Neutrophils containing phagocytosed bacteria were often noted in the ileal lumens of 6- and 24-hour gilts vaccinated with the Troyer strain.

Figure 2.5. Macrophage containing bacteria resembling E. coli (Insert). Slightly rugose bacterial cell walls are in close apposition with phagosome membranes (arrows). Lysosomes (L). From subepithelial area in Peyer's patch dome of 6-hour gilt orally vaccinated with the Troyer strain. Gilt 2086

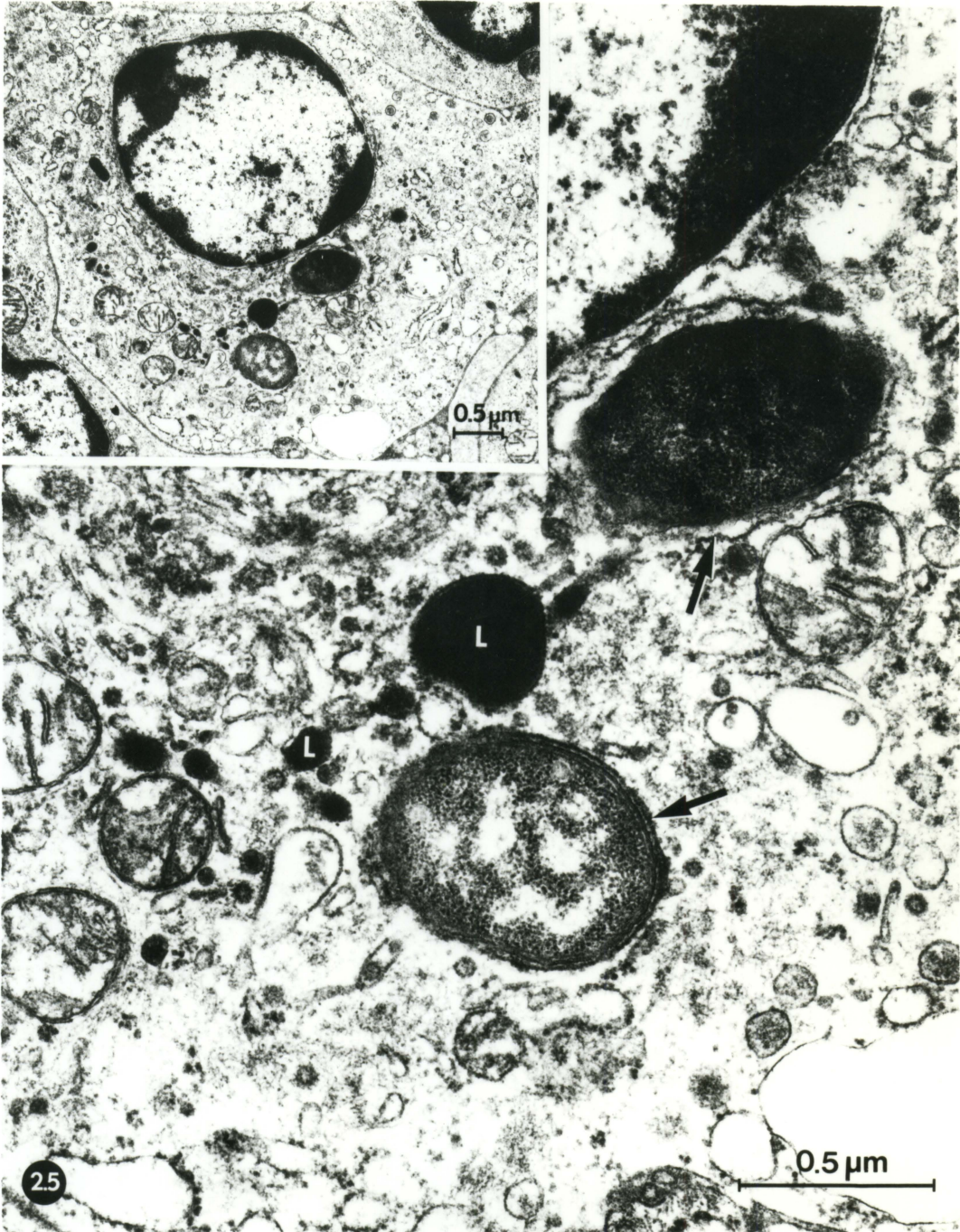
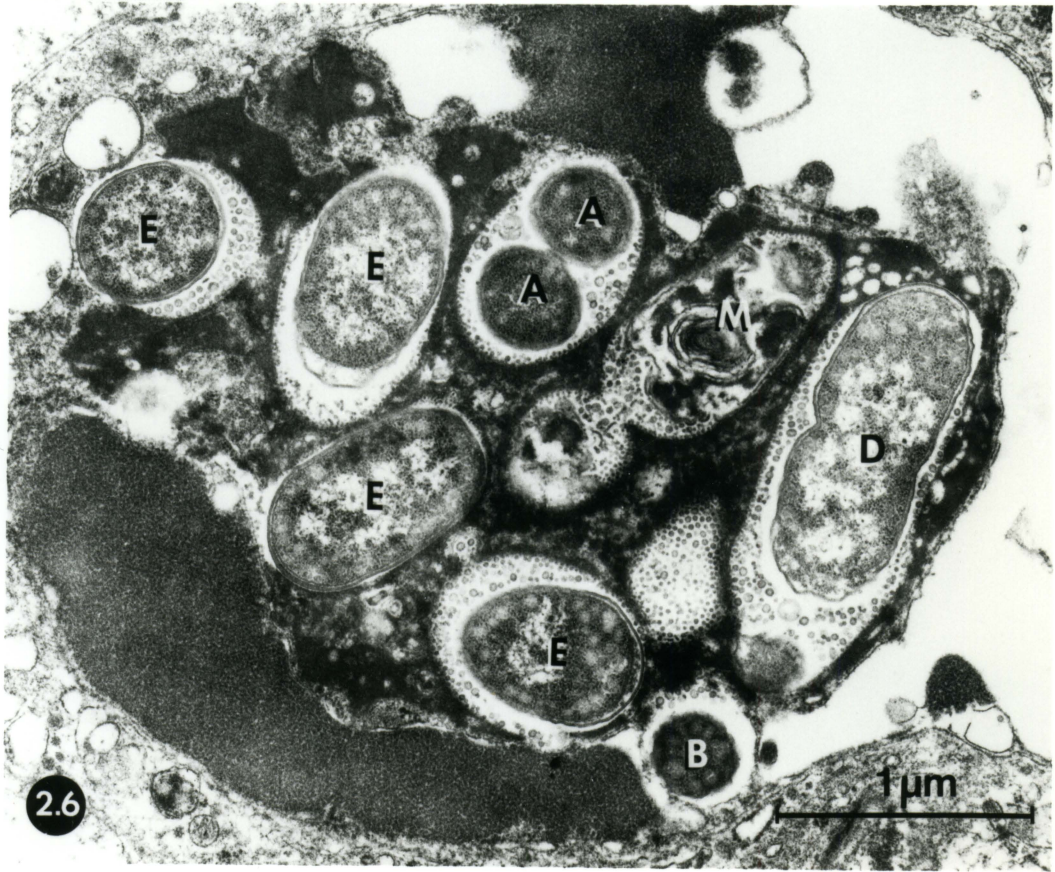


Figure 2.6. Bacteria resembling E. coli (E) associated with necrotic cell. Dividing bacterium resembling E. coli (D), myelin figure inclusion (M), bacteria with indistinct morphology (B), and bacteria with morphology atypical of E. coli (A). From subepithelial area in Peyer's patch dome of 6-hour gilt orally vaccinated with the Troyer strain. Gilt 657



DISCUSSION

Oral vaccination of pregnant gilts with the Troyer strain resulted in an early distribution of this organism throughout the small intestine. Bacterial translocation to the mesenteric lymph nodes in some animals suggests that this strain passed through mucosal epithelium. Mesenteric lymph nodes have been suggested as a site of bacterial interaction with immunocompetent cells (86). Electron microscopy revealed macrophages containing phagocytosed bacteria in Peyer's patches of those gilts fed the Troyer strain 6 hours earlier. Although many phagocytosed bacteria resembled E. coli, it can only be suggested that this is a mechanism by which the Troyer strain activates the secretory immune system. The mode of bacterial entry into Peyer's patches was not evident in this study, but M cells and discontinuity in dome epithelium are suggested portals (94, 127).

In contrast, the K12(K99⁺) strain did not appear to effectively colonize the small intestine (109) or translocate to the mesenteric lymph nodes of orally vaccinated gilts. The inability of this strain to elicit a sufficient secretory immune response may reside in its limited interaction with gut-associated lymphoid tissue (GALT) in the small intestine.

The colon has been shown to respond to mucosal immunization (119, 134). Some investigators have suggested that oral vaccination of swine actually results in immunization of the large intestine (115). It seems feasible to suggest a role for the cecum and colon in secretory immunity, but the relative importance of these sites is difficult to

ascertain. The Troyer strain persisted at these sites for a period of time after its numbers had diminished in the small intestine. Prolonged exposure to GALT in the cecum and colon might possibly enhance the immunogenicity of this strain. The K12(K99⁺) strain was isolated almost exclusively from the cecum and colon. This strain's ability to induce a minor secretory immune response may be the result of interaction with GALT at these sites. Both the Troyer and K12 strains appeared to be rather transient inhabitants of the cecum and colon. Neither strain was isolated from the feces at 48 hours' post-feeding. Kohler has reported isolating the Troyer strain from the feces of sows 2 to 8 days after feeding vaccine (88).

The Troyer strain was not consistently isolated from lymph nodes of gilts 6 hours after vaccination. This finding makes it difficult to evaluate persistence of this strain at these sites. It would be useful to know if viable bacteria are consistently distributed in lymph nodes earlier than 6 hours after vaccination. In addition, it is possible that antigen distribution is not complete at 6 hours' post-feeding. Future studies might be designed to determine distribution of the Troyer strain shortly after vaccination and perhaps at intervals between 6 and 24 hours' post-feeding. Perhaps then, persistence of viable bacteria at these sites might be determined.

The inability to detect rotavirus in the feces of piglets shortly after oral inoculation has recently been attributed to the formation of immune complexes (39). Early in this study, it was hypothesized that a similar phenomenon might occur when animals were fed the Troyer strain

for 3 successive days. This hypothesis was subsequently discounted, and the reasons for failing to recover the Troyer strain from the intestinal tract of sow 1 in the pilot study are unknown. The immune status of this animal was not known. All pilot study animals had been confined in isolation for a period of time prior to vaccination, and exposure to the Troyer strain is unlikely. Gastric acidity, indigenous microflora (8, 9), pancreatic enzymes, and mucus secretion undoubtedly influence the distribution of antigen in the gastrointestinal tract and associated lymphoid tissues. The variable distribution of antigen observed within individual groups of animals may be attributed to these nonspecific environmental factors.

The nature of antigen presentation within Peyer's patches remains unclear (175). Some investigators speculate that macrophages in Peyer's patches process antigen and present it to immunocompetent lymphocytes (10). The present study clearly demonstrates an intimate relationship between macrophages and bacteria within Peyer's patches. However, the importance of this relationship relative to the induction of secretory immunity could not be determined. It would be tempting to suggest that macrophages in Peyer's patches participate in the induction of secretory immunity in orally vaccinated swine. Continual improvements in tissue culture methodology should provide more insight into the cellular interactions within Peyer's patches.

The present study has focused on the distribution and persistence of viable bacteria following oral vaccination. However, this study cannot account for immunogenic moieties that may continue to persist after

viable bacteria are no longer isolated. Salmonella flagellar antigen has been shown to persist in lymph nodes of rats for variable periods of time. Persistence was especially prolonged in lymphoid follicles of immune animals, where antigen was associated with specialized dendritic cells (116, 117, 118). Although Peyer's patches contain dendritic cells (154), their function at this site is unknown (166). Macrophage-associated antigen may remain immunogenic for prolonged periods of time. It appears that antigen is not completely degraded by macrophages but becomes associated with macrophage cell membrane or RNA (168). Some immunogens have been shown to persist in macrophages for at least 3 weeks (169). In another study, fragments of E. coli were immunogenic 2 hours after degradation by macrophages (35). Unfortunately, this investigation was not conducted at longer time intervals. It is conceivable that enterotoxigenic E. coli antigen persists in the mesenteric lymph nodes and/or Peyer's patches following oral vaccination. This antigen may be associated with dendritic cells of the follicles or typical extra-follicular macrophages. Antigen persisting at these sites may continue to provide antigenic drive for precursor IgA cells homing to the mammary gland. This concept would afford one explanation for the prolonged secretion of IgA into the milk of orally vaccinated swine.

CONCLUSIONS

Oral vaccination of pregnant gilts with the Troyer strain results in an early distribution of this organism throughout the small intestine. The numbers of this strain diminish in the small intestine between 6 and 24 hours' post-vaccination; however, they continue to persist in the cecum and colon for at least 24 hours. Bacterial translocation to the mesenteric lymph nodes suggests that the Troyer strain can pass through mucosal epithelium. Peyer's patches appear to be a site of bacterial uptake and/or entry. The present study suggests Peyer's patches and mesenteric lymph nodes as sites where the Troyer strain may activate the secretory immune system. Solitary lymphoid nodules of the intestinal tract must be considered as additional sites of bacterial interaction with the secretory immune system.

In contrast, the K12(K99⁺) strain does not appear to effectively colonize the small intestine or translocate to the mesenteric lymph nodes of orally vaccinated gilts. The inability of this strain to provoke a vigorous secretory immune response may reside in its limited interaction with GALT in the small intestine. The K12(K99⁺) strain can persist in the cecum and colon for at least 24 hours, however.

The present study has shown tissue distribution of the Troyer and K12(K99⁺) strains following oral vaccination of pregnant gilts. Persistence of these two strains along mucosal surfaces was determined. However, several important questions remain unanswered and warrant further investigation. Is the Troyer strain consistently distributed in lymph nodes or is this distribution subject to individual variation? How

long does viable Troyer strain persist in lymph nodes? Do immunogenic moieties persist in lymphoid tissues after viable bacteria are no longer isolated? Do immunocompetent lymphocytes leaving the tonsil and medial retropharyngeal lymph node selectively home to the intestine and/or mammary gland? What is the mechanism of bacterial uptake and/or entry into Peyer's patches? And finally, what role does GALT in the cecum and colon assume in secretory immunity?

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ACKNOWLEDGMENTS

I would like to express sincere thanks to my major professor, Dr. Harley Moon, for allowing me the opportunity to conduct this research. I am certain that his constructive criticism, guidance, and inspiration will be beneficial throughout my professional career.

In addition, I would like to thank Drs. T. T. Kramer, J. F. Pohlenz, and L. H. Arp for their advice while serving on my graduate committee.

I am greatly indebted to Anne Morgan and Gary Fry for their technical support, friendship, encouragement, and good humor during the more difficult times.

Special thanks to Mayo Skartvedt, Jayme Gleason, Karen Schlueter, and John Flickinger for excellent technical support and suggestions. Special thanks also to Leah Oppedal for typing (and retyping) this thesis.

The photography staff at the National Animal Disease Center is to be commended for excellent photography. Support from the librarians and animal supply personnel is also gratefully acknowledged.

Finally, I would like to thank Drs. P. A. O'Berry, G. Lambert, and N. F. Cheville for initially making this opportunity possible.