

PILUS ANTIGEN INCIDENCE AND CONFERRED IMMUNITY OF
WHOLE BACTERINS AND PILUS VACCINES IN GUINEA PIGS
AND PREGNANT GILTS

by

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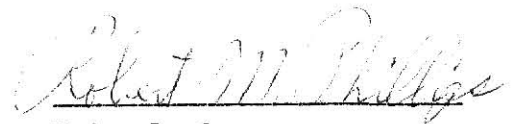
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INCIDENCE OF PILUS ANTIGENS ASSOCIATED WITH
ENTEROTOXIGENIC PRODUCING ESCHERICIA COLI
ISOLATED BY THE KSU VETERINARY
DIAGNOSTIC LABORATORY

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TABLE OF CONTENTS

	<u>Page</u>
Acknowledgements.	ii
List of Tables	iii
Paper I	1
References	10
Paper II.	14
References	23
Paper III	25
References	42
Abstracts	

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LIST OF TABLES

Table		Page
Paper I		
Table 1	Enterotoxin types and pilus antigens detected in all isolates of the study	5
Table 2	Number of isolates per species of animal and types of pili found in each	6
Paper II		
Table 1	Response of guinea pigs in the four E. coli vaccine groups to serotype K87 K88ab purified pili with and without aluminum hydroxide adjuvant and to whole formalized bac-trin with and without aluminum hydroxide adjuvant	20
Paper III		
Table 1	Weight gained	31
Table 2	Intestinal Titers of Challenge E. coli/10 cm. of ileum .	34
Table 3	Circulating antibody Titers of the Dams	36
Table 4	Colostrum Titers - Immediately Post Parturient.	38

THE INCIDENCE OF PILUS ANTIGENS ASSOCIATED WITH
ENTEROTOXIGENIC PRODUCING E. COLI ISOLATED
BY THE KSU VETERINARY
DIAGNOSTIC LABORATORY

INTRODUCTION:

There are several syndromes of enteric disease caused by Escherichia coli. These syndromes have been documented in domestic animals, birds, and man. There are various forms of enteric disease caused by E. coli and range from local enterotoxic to invasive-septicemic.

Pathogenic and nonpathogenic are ways of referring to E. coli that do and do not cause disease respectively. The potential to cause disease "of an enteric nature" is described as enteropathogenic by Netter.² These organisms can be further described as enterotoxigenic or non-enterotoxigenic referring to the ability to product toxins.³

Certain strains of E. coli possess special virulence properties; among these are production of pili and toxins. Bacterial pili or fimbriae are nonflagellar filamentous appendages on the bacterial surface.^{4,5} Pili are made of polymers, or repeating subunits of pilin and consist mainly of protein with a high content of hydrophobic amino acid. The pili, are extremely hydrophobic proteins, and are easily contaminated by other bacterial surface antigens such as outer membrane protein and lipopolysaccharide.⁶ The presence of contaminants makes purification of pili difficult. This has lead to the classification of E. coli pili based on fimbriae referred to as colonization factor antigens I and II on human enterotoxigenic E. coli. The type I pili are described as being 7 nm in width and causing mannose-sensitive agglutination of guinea pig erythrocytes. The 987P strain of animal, E. coli resembles the type I pili morphologically, but cause mannose-

resistant agglutination of human and bovine erythrocytes, making them similar but distinct from those found in^{8,9,10} human intestines. Animal K88 pili strains resemble K99 pili strains of E. coli in that both are plasmid mediated.

The colonization factor or pili of enteropathogenic E. coli found in domestic animals, namely bovine, ovine, and porcine are known as K88, K99, and 987P antigens.^{11,12} It is generally accepted that colonization of the mucosal surface of the small intestine by enterotoxigenic E. coli (ETEC) occurs without tissue invasion and is one of two necessary components in the pathogenesis of diarrhea.¹³ Colonization depends on the ability of the ETEC strain present to adhere to the villus epithelium. This attachment is mediated by the pili. The production and release of heat-labile (LT), or heat-stable (ST), or both, enterotoxins are required for a strain to be enterotoxigenic, and the amount formed is related to the productive potential and population size of the colonizing strain. The LT is reported to cause increased fluid secretion by activation of adenylcyclase in the intestinal mucosa.¹⁴ The mechanism of induction of fluid secretion by ST is unknown but thought to be the same.¹⁵

The sequence of events in enterotoxic colibacillosis is:

1. Proliferation of ETEC in the lumen of the small intestine.
2. Attachment to the villus epithelium by the pili.
3. Production and release of enterotoxins.
4. Induction of excess fluid secretion by the enterotoxins.
5. Net loss of isotonic fluid into the intestinal lumen with subsequent expulsion of liquid feces.

6. Sustained loss of fluid and electrolytes causing dehydration, hemoconcentration, and acidosis often leading to death.¹⁶

Accurate and specific etiologic diagnosis of infectious diarrheal diseases of domestic animals is difficult to obtain. Reasons for this are: Mixed or multiple agents involved; asymptomatic infections with some of the agents; signs are often nonspecific.

The primary objective of the present study was to determine the frequency of the various pili antigens among the ETEC isolated at the Kansas State University (KSU) Diagnostic Laboratory.

Materials & Methods

Test Cultures

A total of 100 E. coli isolated from calves and pigs with diarrheal disease that were collected at the KSU Diagnostic Laboratory were used in this study. The isolates, once in pure cultures were tested for LT by the Y-1 mouse adrenal cell culture test¹⁷ and then tested for ST by the infant mouse assay.¹⁸ The cultures were selected randomly and on the basis of their ability to produce toxin, either ST or LT. The isolates were collected from February 20, 1980 to October 10, 1980 and after they were tested for presence of toxin they were stored on Tryptocase soy agar slants at room temperature in the dark.

Pili Antisera

All the antiserums used in this study were of rabbit origin. The K99 and 987 pilus antisera was purchased from Dr. Paul Glantz, University of Pennsylvania. The K88 antisera was produced by Dr. Robert Phillips, KSU by injection of rabbits, with purified K88 pili. The strains of E. coli used to produce the antisera are K87-K88ab 4-G-7, K87-K88ac

0149, K99-K99 0101 B85, K99 0101 B41, 987-P987-K30, OP987-K35, OP987-K99.

These sera were considered to be monospecific pilus antisera and were used in agglutination tests at dilution of 1:10.

Pilus Antigen Detection

All cultures were inoculated into one ml of Trypticase soybroth, grown for 18 hours at 37°C without shaking, 0.02 ml of the culture was placed on a slide and combined with 0.02 ml of K88 pili antiserum. The slides were then rotated for four minutes to complete the slide agglutination test, and any agglutination recorded. Positive isolates were then tested for agglutination with normal rabbit serum to rule out any false positives from self agglutination.

Isolates that remained K88 negative were reinoculated on trypticase soybroth and incubated for 18 hours at 37°C without shaking and then tested for agglutination with P987 antisera. As before any positives were tested for autoagglutination with normal sera.

Isolates that were negative to both K88 and P987 pili antisera were then inoculated in 2 ml of Minca IS broth and incubated at 37°C for 18 hours without shaking. The Minca broth is a minimal salts media containing Isovitalex developed to enhance K99 pili expression. Following the initial incubation, 0.2 ml was transferred to a new 2 ml tube of Minca broth and the culture was reincubated as before. Upon being passed to fresh broth two times the slide agglutination test for presence of K99 pili was completed and all positive agglutinations rechecked with normal sera.

Results

Enterotoxin Type (Table 1)

Forty of the isolates produced LT, 56 of the isolates produced ST, and 4 isolates produced LT and ST toxins.

Pilus Antigens (Table 1)

Forty-two of the isolates produced K88 pili, 33 isolates were K99 pili producers, and 3 were found to express 987P pili. The remaining 22 isolates did not express pili or agglutinate with the antisera.

Species of Animal (Table 2)

Ten of the isolates came from calves, all of which produced stable toxins. Of these 10 isolates, 8 were positive on the slide agglutination test for production of K99 pilus and 2 did not express pili.

The other 90 isolates all came from pigs, 42 of these were K88 positive. Twenty-five were K99 positive, 3 were 987P positive, and 20 produced no pili.

Table 1. Enterotoxin types and pilus antigens detected.

	K88	K99	987P	no pili
LT	34	3	0	3
ST	7	30	3	16
LT/ST	1	0	0	3

Table 2. Number of isolates per species of animal and types of pili found in each.

	isolates	K88	K99	987	no pili
calves	10	0	8	0	2
pigs	90	42	25	3	20

Discussion

The above results indicate that 56% of the toxin producing isolates produce stabile toxin, 40% produce labile toxin and only 4% produce both toxins. It must be considered that this study was completed on (+) toxin producers and not on all isolates. The results also show that 42% produce K88 pili, 33% produce K99 pili and 3% produce 987 pili. The remaining 22% did not express any pilus production according to the plate agglutination test. It has been shown in other studies that 987 piliated strains of E. coli do not readily produce diarrhea in piglets even when innoculated in very high concentrations.¹⁹ Moon also reports that 987 antigen were not detected until isolates were grown in vivo. This may be responsible for the low number of isolates²⁰ of the 987 group present in this study, as he further indicates the piliated phase tends to grow in the small intestine and the nonpiliated phase in vitro. One can only postulate that 987 is of lesser significance as an enteropathogenic strain due to its rapid ability to change phases from piliated to nonpiliated. This also lends credibility to the immunoflourescent tests which is done on freshly necropsied intestines

and would detect more readily the colonization of villus epithelial cells.¹⁴

Many K99 ETEC are difficult to recognize when grown in vitro due to lack of pilus expression.²² The most sensitive diagnostic method is the use of immunofluorescence of sections or scrapings of the ileum on animals suspected of diarrheal disease due to K99 ETEC.²³ The difficulty in growing these isolates in vitro and getting them to express their pili may explain the preponderance of ST producing E. coli in this study that did not express pili on the slide agglutination test. Studies indicate a high incidence of K99 pili associated with stable toxin production in E. coli.²⁴

Conclusions

This study is consistent with the findings that K99 pili are often associated with diarrheal disease in calves. That 987 is also a cause of disease,²⁵ in calves could not be supported, probably due to too few isolates from calves and in vitro culturing techniques which are not conducive to pilus expression.

This study is also consistent with K88 pilus producing E. coli predominating as the most likely cause of diarrheal disease in pigs, and being associated primarily with labile toxins.²⁶ K99 ETEC are an important cause of enteric disease and while almost all (30 out of 33) produced stable toxins, 3 isolates produced labile toxins. All of the 987 pilus producing ETEC in this study were found as isolates of pigs, but this only comprised 3% of all isolates indicating difficulty in pili expression as well as low virulence.

Pilus antigens can be used in identifying ETEC infections because they are virulence attributes and because they occur most commonly on ETEC.

The incidence of each serotype is significant in that it may aid in predicting the possible protection that could be expected from a pili vaccine containing any or all of these serotypes.

SUMMARY

Enterotoxigenic *Escherichia coli* isolated from pigs and calves were examined for the presence of K88, K99 or 987P pili. A total of 42% possessed K88 pili, 33% produced K99 pili and 3% produced 987P pili. The remaining 22% did not express any pilus production according to the plate agglutination test. The K88 possessing E. coli were isolated primarily from swine and the K99 came from calves with severe diarrhea.

REFERENCES

1. Kohler, E.M.: Neonatal Enteric Colibacillosis of Pigs and Current Research on Immunization. JAVMA. 170:588-591, 1978.
2. Neter, E.: Enteropathogenicity of E. coli. Amer. J. Dig. Dia. 10:883-886, 1965.
3. Moon, H.W.: Pathogenesis of Enteric Diseases Caused by Escherichia coli: Infect. Immun. 179-211, 1972.
4. Houvink, A.L. and W. Van Iterson: Electron Microscopic Observations on Bacterial Cytology II. A study of Flagellation. Biochem. Biophys. Acta. 5:10-44, 1950.
5. Ottow, J.C.G.: Ecology, Physiology and Genetics of Fimbriae and Pili. Ann. Rev. Microbiol. 29:79-108, 1975.
6. Brinton, C.C.: The Structure, Function, Synthesis, and Genetic Control of Bacterial Pili and a Model for DNA and RNA Transport in Grain Negative Bacteria. Trans. N.Y. Acad. Sci. 27:1003-1054, 1977.
7. Salit, J.E. and E.C. Gotschlich. Haemagglutination by Purified Pili-type I Escherichia coli pili. J. Exper. Med. 146:1169-1181, 1977.
8. Eisenstein, B.I., I. Ofek and E.H. Beachey. Interference with the Mannose Binding and Epithelial Cell Adherence of Escherichia Coli by Sublethal Concentrations of streptomycin. J. Clin. Invest. 63:1219-1228, 1979.
9. Duguid, J.P., J.W. Smith, G. Demster and P.N. Edmunds. Nonflagellar Filamentous Appendages (fimbriae) and Haemagglutination Activity

- in Bacterium Coli. J. Pathol. Bacteriol. 70:335-348, 1955.
10. Engvall, E., and P. Pearlmann. Enzyme-Linked Immunosorbent Assay. ELISA III Quantitation of Specific Antibodies by Enzyme-Labelled Antiimmunoglobulin in Antigen Coated Tubes. J. Immunol. 109: 129-135, 1972.
 11. Moon, H.W., B. Nagy, R.E. Isaacson, and J. Oskov. Occurrence of K99 Antigen on Escherichia Coli Isolated From Pigs and Colonization of Pig Ileum by K99+ Enterotoxigenic E. coli From Calves and Pigs. Infect. Immun. 15:614-620, 1977.
 12. Moon, H.W., S.C. Whipp and S.M. Skartvedt. Etiologic Diagnosis of Diarrheal Disease of Calves: Frequency and Methods for Detecting Enterotoxin and K99 Antigen Production by Escherichia Coli. Am. J. Vet. Research 37:1025-1029, 1976.
 13. Moon, H.W. Mechanisms in the Pathogenesis of Diarrhea; a Review. J. Am. Vet. Med. Assoc. 172:443-448.
 14. Evans, D.L. Jr., L.C. Chen, and G.T. Curlin, et al. Stimulation of Adenyl Cyclase by Escherichia coli. Enterotoxin. Nature (New Biology) 236:137-138, 1972.
 15. Whipp, S.C.: Studies on Escherichia coli Heat Stable Enterotoxin in Proceedings. International Pig Vet Society. 1976.
 16. Kohler, E.M.: Pathogenesis of Neonatal Enteric Colibacillosis of Pigs. JAVMA. 160:574-582, 1972.
 17. Sack, D.A., and R.B. Sack. Test for Enterotoxigenic Escherichia coli Using Y1 Adrenal Cells in Miniculture. Infec. Immun. 11:334-336, 1974.
 18. Dean, A.G., Y.C. Ching, R.G. Williams, and L.B. Harden. Test for

- Escherichia coli* Enterotoxin Using Infant Mice: Application in a Study of Diarrhea in Children in Honolulu. *J. Infect. Dis.* 125: 407-411, 1972.
19. Isaacson, R.E.: Factors Affecting Expression of the *Escherichia coli* Pilus K99, *Infect. Immun.* 28:190-194, 1980.
 20. Moon, H.W., E.M. Kohler, R.A. Schneider, and S.C. Whipp. Prevalence of Pilus Antigens, Enterotoxin Types and Enteropathogenicity Among K88 Negative Enterotoxigenic *Escherichia coli* from Neonatal Pigs. *Infect. Immun.* 27:222-230, 1980.
 21. Hadad, J.J. and C.L. Gyles: Detection of Bovine Enteropathogenic *Escherichia coli* by Indirect Fluorescent Antibody Technique. *Am. J. Vet. Res.* 39:1651-1655, 1978.
 22. Guinee, P.A.M., J. Veldkamp, W.H. Jansen: Improved Minca Medium for Detection of K99 Antigen in Calf Enterotoxigenic Strains of *Escherichia coli*. *Infect. Immun.* 15:576-678.
 23. Lariviere, S., R. Lallier, and M. Morin. Evaluation of Various Methods for the Detection of Enteropathogenic *Escherichia coli* in Diarrheic Calves. *Am. J. Vet. Res.* 40:130-134, 1979.
 24. Orskov, F., I. Orskov, H.W. Smith, and W.J. Sojka. The Establishment of K99, a Thermolabile Transmissible *Escherichia coli* K Antigen, Previously called "KCO" Possessed by Calf and Lamb Enteropathogenic Strains. *Acta. Pathol. Microbiol. Scand. Sect. B* 83:31-36, 1975.
 25. Smith, H.W., and M.M. Huggins: The Influence of Plasmid Determined and Other Characteristics of Enteropathogenic *Escherichia coli* on Their Ability to Proliferate in the Alimentary Tracts of Piglets, Calves and Lambs. *J. Med. Microbiol.* 11:471-492, 1978.

26. Nagy, B.H., W. Moon, and R.E. Isaacson. Colonization of Porcine Intestine by Enterotoxigenic *Escherichia coli*: Selection of Pileated Forms in Vivo, Adhesion of Piliated Forms to Epithelial Cells in Vitro and Incidence of a Pilus Antigen Among Porcine Enteropathogenic *E. coli*. *Infect. Immun.* 16:344-352, 1977.

A COMPARISON OF ESCHERICHIA COLI BACTERIN VACCINE
AND PURIFIED PILI VACCINE IN GUINEA PIGS

G.L. Baughman and R.M. Phillips

SUMMARY

Escherichia coli serotype K87-K88ab was used to produce a whole formalized bacterin and a pili vaccine. The pili was purified by homogenization, ammonium sulfate precipitation, and DEAE-Sephadex column chromatography. The vaccine was injected into 4 groups of guinea pigs with and without aluminum hydroxide adjuvant. The antibody titer was determined by serial dilutions.

The higher titers of circulating antibody in guinea pigs injected with purified pili vaccine and aluminum hydroxide, led to the conclusion that the pili was a superior means of stimulating antibody response.

INTRODUCTION

In swine, production of neonatal diarrhea stems from ingesting Escherichia coli with the ability to produce enterotoxin and attach to the gut wall epithelial cells.¹ The toxin increases the adenylyl cyclase activity in the epithelial cells resulting in severe fluid loss.² Portions of the intestine, however, are affected more than others. The colon seems to be little affected by the toxins, while the small intestine is severely affected. For disease to occur, the E. coli must remain in the upper portion of the small intestine by attachments.³ Pili, a nonflagellae hairlike appendage of bacteria make the attachment possible.⁴ Somatic pili differ from the sex pili necessary for conjugal DNA transfer.

Somatic pili are found covering the bacteria at all locations and are more numerous than the sex pili. Pathogenicity of E. coli requires both toxin production and pili for attachment.

The principle of a pili vaccine is to produce antibodies in the host animal to eliminate bacterial attachment to the gut wall epithelial cells and decrease colonization of the small intestine. Such a vaccine would have the advantage of preventing pathogenic E. coli from spreading within the herd as in the "milk vaccine".

Our objectives were to compare the circulating antibody production to the pili antigen consisting of assemblies of identical protein subunits and the antibody production of the killed E. coli formalized bacteria of serotype K38ab. Alhydrogel adjuvant was used with each type of vaccine to enhance the immune response.

MATERIALS AND METHODS

E. coli strain. The strain used to produce both the purified pili, and the bacterin was K87-K88ab, obtained from Dr. Paul Glantz, Pennsylvania State University. This strain was positive on the infant mouse test and produced heat-stable enterotoxin. Production of the piliated phase was identified by plate agglutination of 24-hour, small-mucoid colonies grown on blood agar.

Growth of the Bacteria. The small colonies just described were picked from blood agar and inoculated into trypticase soy broth (TSB) and incubated 24 hours at 37C without shaking. These cultures were inoculated into 1 liter of TSB and incubated 18 to 20 hours at 37C with shaking.

Purification of the Pili. The pili was purified by Isaacson's method, which is briefly described.

The bacteria were removed from the media by centrifugation at 3200 x g for 30 minutes.

The precipitated bacteria from each liter were resuspended in 20 ml. of 0.05 M sodium phosphate buffer, pH 7.2 containing 1.0 M sodium chloride.

The bacteria were then homogenized at #7 setting in a Sorvall Omnimixer for 30 minutes at 4C.

Bacteria were removed by centrifugation at 17000 x g for 10 minutes at 4C, leaving the pili in suspension.

Homogenization and centrifugation were used a second time on the precipitated bacteria, the two supernatants were pooled and then stored at -20C until used.

Ammonium Sulfate Precipitation. 10.6 g/100 ml of ammonium sulfate was added to the pooled supernatants with constant stirring for 30 minutes at 4C.

The insoluble precipitate is removed by centrifugation at 20,000 x g for 10 minutes.

11.3 g/100 ml of ammonium sulfate is again added with constant stirring and the insoluble precipitate is collected by centrifugation and resuspended in PBS. This solution is then dialyzed overnight against PBS until the ammonium sulfate is removed.

DEAE - Sephadex Column Chromatography. The dialyzed solution is applied to the DEAE-sephadex A-50 column (215 x 40 cm.) after the column has been equilibrated and developed with 300 ml of PBS pH 7.2.

Following the sample application, 300 ml of PBS was applied to the column and the pili were recovered in the void volume.

The purified pili were collected and concentrated to 10 ml with PBS, then stored at -70C until ready to use.

Preparation of the Whole Cell Vaccines. The bacteria were grown as described above in TSB. Plate agglutination was used to determine the presence of the pillar antigen. The media were plated out to determine the titer of organisms present after 18 to 24 hours' incubation. Once the titer was determined, the bacteria were centrifuged at 3200 x g for 30 minutes and resuspended in 1% formalin and saline at a concentration of 9.4×10^7 μ g. per milliliter. This solution was allowed to stand at room temperature 48 hours. To ascertain that all organisms were killed, we plated the solution out on blood agar and incubated it at 37C for 24 hours--with no growth.

Vaccination Procedure. The 15 guinea pigs used were divided into five groups of three with a control group. The guinea pigs were injected twice subcutaneously 17 days apart over the shoulder. The first group was given 0.8 ml of aluminum hydroxide adjuvant plus 50 micrograms of purified K88ab pili in 0.8 ml PBS for a 1.6 ml dose of vaccine; the second group was given 0.8 ml containing 50 micrograms of K88ab pili; the third group was given 0.8 ml of aluminum hydroxide plus 1 ml of culture containing 9.4×10^7 organisms; the fourth group was given 1 ml of culture with 9.4×10^7 organisms. The control guinea pigs were not injected.

Collection of Blood. Twenty-one days after the second injection, each guinea pig was bled from the heart.

Plate Agglutination Procedure. Two-fold dilutions with 0.05 ml of serum in 0.05% formalized saline were made. To each dilution was added 0.05 ml of culture containing 4.7×10^6 organisms. The plate was then rotated 5 minutes before the test was read and the results recorded.

Results. The importance of the K88 antigen, which enables enterotoxigenic E. coli to attach to the epithelial cells, has been expressed by other investigators.^{6,7,8} The pili allows close association of the toxin producing E. coli with the cells of the intestine to cause enteric disease and economic losses. Control of this disease by husbandry and antimicrobial agents has not been effective. Even the results of various immunological approaches have not been gratifying.

The results of this study clearly indicate much higher levels of circulating antibodies to the purified pili antigen than to the whole

bacterin (Table 1). The titers of circulating antibodies in pili-vaccinated guinea pigs were at least twice the level found in pigs vaccinated with the whole-cell vaccine. An aluminum hydroxide adjuvant enhances the immune response to the purified pili (compare groups 1 and 2). But no difference in the immune response to the adjuvant appeared in groups 3 and 4, vaccinated with the whole bacteria.

The clinical response of the guinea pigs to administration of the vaccine was clear. None of the animals in group 1 or 2 showed more than the expected discomfort from the injections. Response to the bacterin injected by groups 3 and 4 was marked. Groups 3 and 4 guinea pigs exhibited endotoxic shock, increased respiration, and squealing 15 to 20 seconds after injection with hyperesthesia. Some appeared weak, while others underwent repeated toxic contraction of limbs and trunk and skeletal muscles. None required treatment, and all recovered uneventfully in 10 to 15 minutes.

Both from clinical and immunological aspects, the pili antigen appears to be a superior way to stimulate immune response. The present methods of pili purification need to be improved to make the vaccine economically feasible.

Table 1. Response of guinea pigs in the four *E. coli* vaccine groups to serotype K87 K88ab purified pili with and without aluminum hydroxide adjuvant and to whole formalized bacterin with and without aluminum hydroxide adjuvant.

		Quantity of vaccine/ injection	Titer of anti- bodies produced
<u>Group 1</u>			
Purified pili & A10H adjuvant Guinea Pig	#1 #2 #3	50 micrograms of pili and 40 micrograms aluminum hydroxide adjuvant	1:32 1:16 1:16
<u>Group 2</u>			
Purified pili Guinea Pig	#4 #5 #6	50 micrograms pili	1:2 1:2 1:8
<u>Group 3</u>			
Formalized whole organism & A10H Adjuvant Guinea Pig	#7 #8 #9	9.4×10^7 organism in 1 ml saline + 40 mg aluminum hydroxide adjuvant	1:1 1:1 1:1
<u>Group 4</u>			
Formalized whole organism Guinea Pig	#10 #11 #12 #13 #14 #15	9.4×10^7 organism 1 ml saline 0 0 0	1:1 1:1 0 0 0 0
<u>Control Group</u>			

DISCUSSION

To properly assess the response of circulating antibodies to the purified pili and the whole bacterin, a review of the essential features of antigenicity is necessary. To be antigenic, molecules must have three qualities--large size, rigidity, and be chemically complex. Macromolecules of complex structure like proteins⁹ and pili are considerably better antigens than simple large polymers with identical repeating subunits. So lipids, carbohydrates, nucleic acids, and monoamino acid polymers are relatively poor antigens. The outer covering of E. coli is composed of lipopolysaccharide and lipoprotein, components that do not make the best antigens. Proteins of the pili and the nature of the cell¹⁰ wall of the E. coli organism make both relatively rigid and, in that respect, good antigens. Considering the complexness of the pili and whole bacterin, the protein chain of the pili has readily available surface areas against which immune responses tend to be directed and with which antibodies tend to bind. The interchanging components of lipopolysaccharide and lipoprotein¹¹ of the cell wall make recognition of surface areas or antigenic determinants more difficult, which leads to less antigenicity, and less cross-reactivity of the various strains of the same bacteria. The E. coli with no pili stimulate little immune response and low protection even among similar strains.

Our findings tend to confirm those ideas. The excellent antigenic properties of the purified pili, displayed as greater circulating antibody titer, conclusively indicates the advantages it has as an antigen. These findings confirm that it is a desirable instrument of immunization.

It is extremely risky to extrapolate between different species of animals, but the immunologic responses and the mild clinical reaction of the animals indicate exciting possibilities for the purified pili as vaccination for pregnant swine. The possibility for abortion from whole bacterins makes the pili type vaccine even more attractive. The lipopolysaccharide components, also called "endotoxin" in the cell wall of whole bacterins, has been incriminated by Wilson.¹² Use of live whole bacterins has been encouraged by some investigators¹³ but that presents the problem of propagating virulent bacteria on the farm, which could be avoided with purified pili. Details must be worked out before the pili vaccine becomes available. The purification process must be refined before production of a vaccine will be economically feasible.

REFERENCES

1. Moon, H.W., D.K. Sorenson and J.H. Sautler. 1966. *Escherichia coli*. Infection of the Ligated Intestinal Loop of the Newborn Pig. *Am. J. Vet. Res.* 27:1317-1325.
2. Kanlor, Harvey S., Pearl Tao and Charlene Wisdon. 1974. Action of *Escherichia coli*. Enterotoxin: Adenylate Cyclase. Behavior of Intestinal Epithelial Cells in Culture. *Infection and Immunity*. 9:1003-1010.
3. Jones, G.W. and J.U. Rutler. 1972. Role of K88 Antigen in Pathogenesis of Neonatal Diarrhea Caused by *Escherichia coli* in Piglets. *Infect. Immun.* 6:918-927.
4. Brinton, C.C., Jr. 1959. Non-flagellar Appendages of Bacteria. *Nature (London)* 183:782-786.
5. Isaacson, R.E. 1977. K99 Surface Antigen of *Escherichia coli*: Purification and Partial Characterization. *Infect. Immun.* 15: 272-279.
6. Bertschinger, H.V., H.W. Moon and S.C. Whipp. 1972. Association of *Escherichia coli* with the Small Intestinal Epithelium. *Infect. Immun.* 5:595-605.
7. Jones, G.W. and J.M. Rutler. 1973. Protection Against Enteric Disease Caused by *Escherichia coli*. A Model for Vaccination with a Virulence Determination. *Nature (London)* 242:531-532.
8. Stirne, S., F. Orskov, I. Orskov and B. Mansa. 1967. Episome Carried Antigen K88 of *Escherichia coli*. II. Isolation and Chemical Analysis. *J. Bacteriology*. 93:731-739.

9. Sela, M. 1969. Antigenicity: Some Molecular Aspects. Science, 166:1365-1374.
10. Davis, B.D., R. Dulbecco, H.N. Eisen, H.S. Ginsberg, B.W. Wood and M. McCarty. 1973. Microbiology. Second Edition. pp. 24-25.
11. Tizard, I.R. 1977. Veterinary Immunology by W.B. Saunders Company. Chapter 3. Antigens and Antigenicity. pp. 25-26.
12. Wilson, M.R. Vaccination Against Porcine Neonatal Diarrhea in Proceedings. Mini-symposium on Neonatal Diarrhea in Calves and Pigs. Vet. Infect. Diag. Orga., Univ. Saskatchewan, Canada. 1976. pp. 52-57.
13. Kohler, E.M., Cross, R.F., Bohl, E.H.: Protection Against Neonatal Enteric Colibacillosis in Pigs Suckling Orally Vaccinated Sows. Am. Journal Vet. Res. 36:757-764. 1975.

VACCINATION WITH CRUDE PILI AND WHOLE CELL
BACTERINS IN PREGNANT GILTS TO CONFER IMMUNITY
TO OFFSPRING: A COMPARATIVE STUDY

INTRODUCTION:

Colostrum immunity against K88, K99, and 987 pili has been shown to prevent severe diarrhea and death in suckling piglets challenged with homologous strains of pili producing enterotoxigenic Escherichia coli (ETEC).^{1,2,3} Colonization of the mucosal epithelial cells of the surface of the small intestine is necessary to provide for absorption of toxin.^{4,5} This colonization is mediated by pili which are nonflagellar, hairlike appendages of identical protein subunits sequentially arranged.⁶ These so called K-antigens are quite different and distinct from the polysaccharides that make up the capsule of bacterial organisms. These pili may be present or not, depending on growth conditions.⁷ The ability to clone for piliation has allowed growth, concentration, and pili purification in quantities sufficient for identification and immunization.⁸ The objectives of this study were:

To confirm that pregnant dams vaccinated with a crude pili preparation of K88, K99, and 987 will confer protection to suckling pigs against diarrheal disease following challenge with those respective strains.

To determine the relative serum antibody titers produced as a result of the crude pili vaccine, and to compare these titers with those of gilts vaccinated with a whole cell formalized bacterins using the same strains.

To determine the colostral titers by vaccinated animals and control animals.

To determine the relative numbers of K88, K99 and 987 E. coli found in the small intestines of these piglets following intragastric challenge.

Materials & Methods

Pregnant gilts were either vaccinated subcutaneously behind the ear with a suspension of: (1) a Escherichia coli K88, K99 and 987 pili, (2) a whole-cell formalin killed bacterin (multiple strains as above), or were left as non-vaccinated controls. After birth the piglets were allowed to suckle their dams for 12 to 16 hours and then were challenged orally with one of the three enterotoxigenic strains above. Colostrum was milked from each of the sows immediately postparturition to determine colostral antibody titers against the same three strains mentioned earlier.

Challenge strains of E. coli and inocula

Four strains of ETEC were used to prepare the vaccine in this study and were also used as challenge organisms. The strains used were as follows: (0149:K91:K88ac, 08:k87, K88ab, 0101:K30:k99NM, 09:K103, 987p:NM). These strains were obtained from Dr. Paul Glantz, Pennsylvania State University. The K88ac and K88ab strains were positive in the Y-1 adrenal cell culture assay⁹ and produced heat-labile enterotoxin. The K99 and 987p strains were positive in the infant mouse test¹⁰ and produced heat-stable toxins. The production of piliated phase was

identified by plate agglutination of 24-hour small mucoid colonies grown on blood agar.¹¹ Colonies containing cells agglutinable by monospecific antisera were picked from blood agar and inoculated into 10 ml tubes of Trypticase Soy Broth (TSB) containing .25 percent Dextrose and incubated overnight at 37°C without shaking. These cultures were inoculated into 1 liter flasks of TSB and incubated 18 to 20 hours at 37°C with shaking. This procedure was used for all strains with the exception of K99 which was grown on TSB with .125 percent Dextrose¹² and the 1 liter flasks were incubated 18-20 hours but without shaking.

The bacteria were harvested by centrifugation at 3200 x g for 30 minutes and resuspended in 0.05M sodium phosphate buffer (PBS) pH 7.2, containing 1.0 M sodium chloride, using 20 ml of buffer, for each liter of culture concentrated.

The bacteria were then homogenized at #7 setting in a Polytron mixer for 5 minutes, then pelleted by centrifugation at 10,000 x g for 10 minutes at 4°C. The bacteria were removed leaving the crude pili in solution. Homogenization and centrifugation were repeated on the bacteria precipitate, and the salt dialyzed out of the supernatant using phosphate buffer solution. The two supernatants were pooled and stored at -20°C until ammonium sulfate precipitation was done.

Ammonium Sulfate Precipitation

Ammonium Sulfate (10.6 gms/100 ml) was added to the pooled supernatants with constant stirring for 30 minutes at 4°C.

The insoluble precipitate was removed by centrifugation at 20,000 x g for 10 minutes to purify the pili by removing larger cell wall debris.

Ammonium Sulfate (11.3 gms/100 ml) was again added with constant stirring in the cold and the insoluble precipitate was collected by centrifugation and resuspended in PBS. This solution was then dialyzed overnight against PBS until the ammonium sulfate was removed. Lowry protein determinations were run on the solution and then stored at -70C until ready to use.

Preparation of the Whole Cell Cultures

The bacteria were grown as described above in TSB. Plate agglutination was used to determine the presence of the pillar antigen. The bacteria were plated out to determine the number of organisms present after 18 to 24 hours incubation. Once the organism number was determined, the bacteria were centrifuged at $3200 \times g$ for 30 minutes and resuspended in .2% formalin and saline. This suspension was allowed to stand at room temperature 48 hours. To ascertain that all organisms were killed, they were inoculated on blood agar and incubated at 37C for 24 hours with no growth.

Preparation of the Whole Cell Vaccine

The whole cell bacterin like the crude pili vaccine contained the 4 serotypes K88ab, K88ac, K99 and 987. Bacterial suspensions contained 2.4×10^9 organisms of K99 and 987p. The K88ab and K88ac contained 1.2×10^9 organisms for a total of 2.4×10^9 organisms for the K88 serotype collectively. Each injection contained 7.2×10^9 organisms/ml and was prepared using 3 ml of K99 and 987p, and 1.5 ml of K88ab and K88ac for a total of 9 ml plus 5 ml of aluminum hydroxide used as the adjuvant.

Vaccination Procedure

At 4 weeks prior to farrowing, bred gilts were purchased from

Kansas State University Swine Research and were identified and assigned to one of 3 groups. The gilts were given subcutaneous injections of 10.10 ml of crude pili, 14 ml of whole cell bacterin, or no injection (for control group). The first injection was given at 4 weeks prior to farrowing followed by a second injection at 1 week prior to farrowing.

Trivalent Vaccine Preparation

The crude pili preparation was brought out from -70C storage and quickly thawed. Using the known concentration of each serotype, K88ab, K88ac, K99, and 987p a trivalent crude pili vaccine was prepared. This contained 3 mg of K99 and 987p, and 1.5 mg of K88ac and K88ab to form a resulting solution of 9 mg total crude pili per injection. This was suspended in 5.10 ml of .2% formalin and saline. To this was added 5 ml (250 µg) of aluminum hydroxide per injection. The total injection was 10.10 ml.

Pilus Antibody Titers

Blood was drawn from each gilt immediately prior to vaccination at 4 weeks prior to farrowing, at one week prior to farrowing and on the expected farrowing date. Two fold dilutions with 0.05 ml of serum in 0.05% phenolized saline were made. The plate agglutination test was done using serotype K88ab, K88ac, K99, and 987p and using standardized suspensions of bacteria. These suspensions were standardized using spectrophotometry at a 90 absorbance reading with a wavelength of 600 nm.

Colostrum samples were collected just prior to challenge, clarified by centrifugation at 95,000 x g and stored at -70C. Pilus antibody titers were determined by plate agglutination of pilated bacteria standardized as described above.

All observations and measurements were performed with samples coded to avoid subjective bias in laboratory samples.

Experimental Infections & Observations

At parturition each piglet was identified by ear notching. Piglets were then actively aided in receiving colostrum. The piglets were allowed to suckle for 8 hours and then were challenged with a single strain of organisms. Immediately prior to the challenge each pig was weighed, and any weighing less than 800 grams was eliminated from the experiment. Any pigs in excess of 12 per litter were also eliminated from the experiment. The challenge inoculum consisted of 20 ml of fresh TSB containing 2 ml of the appropriate challenge strain. All pigs in the litter received the same strain. Each pig was weighed daily and recorded along with information on diarrhea, death and general appearance. From each litter beginning on day 5 post-challenge, the pig that had gained the least or lost the most weight was sacrificed, and a 10 cm portion of the ileum was removed 10 cm anterior to the ileocecal junction to determine the number of viable challenge organisms at the site. All pigs were given a "complete necropsy" and gross lesions were recorded, as well as tissue from the liver, kidney, lung, and intestine saved for histopathological study to confirm lesions consistent with enterotoxigenic colibacillosis.

In each group of 3 gilts one litter received K88ab challenge organisms, one received K99 and one received 987p organisms. The groups were kept separate in isolation stalls at the KSU Clinical Science Building at the College of Veterinary Medicine. The gilts were maintained in standard hog crates with heat lamps with divider

screens to prevent intermingling of the piglets.

Results

The results of this study are based on four parameters: weight gained or lost by the piglets, intestinal titer of challenge E. coli per 10 centimeters of ileum, circulating antibody titers of the gilts and colostrol titers of the gilts. Clinical records were kept of appearance and relative amounts of diarrhea, however as these are subjective data they will be treated as such. Necropsys were completed on each piglet and were to rule out death or disease other than colibacillosis.

Table 1. Weight Gained in Challenged Pigs

Vaccine Group	K88	Challenge organism K99	987	Challenge Group Ave. of Weight gained
Crude pili gilts	16%	31%	20%	22.33%
Bacterin gilts	20%	15%	27%	20.66%
Control gilts	13%	22%	12%	15.67%

The data on the weights, recorded daily on each pig was used to determine the percent that each pig gained or lost from birth to necropsy on or before day 5. These weights were used to arrive at an average for each litter which are summarized in Table 1. In the 3 litters vaccinated with the trivalent crude pili, the K99 challenge litter showed no diarrhea clinically at any time which may be reflected in the weight gained by this group. The K88 challenge litter had no pigs

with diarrhea. The 987 challenge litter displayed no signs of colibacillosis at any time during the challenge.

The 3 litters vaccinated with the whole formalized trivalent bacterin, postchallenge the K99 litter appeared to be depressed. This litter seemed to appear as thin gaunt pigs that didn't vigorously nurse or gain well. The K88 challenge litter had four pigs with an orange pasty diarrhea which developed over the first two days postchallenge. The other pigs in the litter were only mildly affected with diarrhea for the first day. The 987 challenge litter had some pigs with diarrhea and others without diarrhea. Those affected in this litter had diarrhea for only one or two days and were not affected the last three days of the 5 day test period. The diarrhea again was pasty and orange not very fluid in nature.

In the Control group, the K99 challenge litters were depressed within a few hours postchallenge. These pigs became thin and possessed a very fluid white diarrhea. This litter remained depressed and gaunt throughout the test. The second day the most severely affected pig was almost comatose when necropsied. The litter challenged with K88 all displayed diarrhea but were not as depressed as the K99 litter. The nature of the diarrhea was semi-fluid yellow in color, and lasted 2 days. The 987 litter all were depressed by the following day post challenge and evidence of a yellow pasty diarrhea was found on the pigs. This litter did not remain depressed but did not do well, and appeared thin throughout the test period. The percent weight gained from birth recorded in Table 1 is listed according to challenge organism and type of vaccine used on each respective litter. The challenge group averages are seen in the far right hand columns. The crude pili

group average indicates a 6.66% greater weight gained in the 5 day test period, than the control group. The whole bacterin challenge group gained 4.99% more than the control group, while a 1.67% difference is seen between the crude pili group and the whole bacterin groups. Statistical analysis of the weights of each piglet showed a significant relationship between weight gained on Day 2 and Day 3 and the number of E. coli present in the intestine. The analysis was completed using alpha at the .05 level. Day 1 weights are the birth weight so Day 2 and Day 3 weights reflect the effects of the challenge. Day 4 and Day 5 weights did not significantly correlate with E. coli numbers, due to elimination of the most severely affected piglets from the test by necropsy, and those who could physiologically compensate for the pathogenic organism.

Data pertaining to the titers of challenge E. coli isolated from the 10 centimeter sections of ileum are summarized in Table 2. The groups were analyzed recording the number of organisms plated out following serial dilution. These values are seen in the table showing the range and the arithmetic mean of each challenge litter. The composite average for the three litters in each challenge group (designated according to type of vaccine used) are shown in the challenge group average column at the far right of Table 2.

The challenge group average for the control group was 7.73×10^8 organisms for the 10 cm section of ileum. The whole bacterin group had 1.57×10^8 organisms, indicating 4.92 times more organisms in the control group than the bacterin group. The crude pili group had 1.15×10^8 organisms, indicating 6.72 times more organisms in the control group than

Table 2. Intestinal Titers of Challenge E. coli/10 cm of ileum

Vaccine Group	Challenge Organism			Challenge Group Average
	K88	K99	987	
Crude Pili	Range Arithmetic Mean	1×10^4 - 11×10^8 2.1×10^6	2×10^4 - 14×10^8 2.2×10^8	1.9×10^4 - 6×10^8 1.25×10^8
				1.15×10^8
Bacterin	Range Arithmetic Mean	2×10^6 - 11×10^8 2.33×10^8	1×10^6 - 8×10^8 2.2×10^8	1×10^5 - 9×10^7 2.3×10^7
				1.57×10^8
Control	Range Arithmetic Mean	4.5×10^6 - 4×10^9 9.0×10^8	7×10^4 - 4×10^9 6.90×10^8	3.1×10^5 - 4×10^9 7.4×10^8
				7.7×10^8

this group. These values also show there to be 1.37 times more organisms present in the 10 cm section of ileum in those vaccinated with whole bacterin than those vaccinated with the crude pili.

Using statistical methods to analyze the E. coli numbers present in the 10 centimeter section of ileum, there was determined to be a significant difference between various litters, or vaccination groups. There was also determined to be significant difference between the types of vaccine used. These observations are valid for alpha at the .001 level. As can be seen from the values in Table 2 the most significant difference is between the control group and the two vaccine groups. Although there was a difference between weights gained, and the number of E. coli present in the intestine, there was not in this study, a significant difference statistically between the crude pili preparation and the whole bacterin vaccine using a .05 alpha level.

Circulating Antibody Titers of the Dams

These titers represent the titers taken three times during the study. The first serum samples were taken as "prevaccination serum" samples just prior to giving the first injection. The second samples were taken immediately prior to the second injection 14 days later and will be referred to as "First injection titers". The last samples were taken as close to farrowing as possible 7 days following the second injection after the gilts were in the farrowing crates and will be called "Second injection titers". These titers are summarized in Table 3 below.

All three gilts in the crude pili group responded with four-fold rises in titer to the K88 antigen on the first injection, a titer that remained at that level following the second injection. Two of the gilts in this group responded with two-fold rises in titer following both the

Table 3. Circulating Antibody Titers of the Dams

Vaccine Group	Dam No.	Prevaccination			First Injection			Second Injection		
		K88	K99	987	K88	K99	987	K88	K99	987
Crude pili	27-6	1:16	1:16	1:16	1:128	1:128	1:8	1:128	1:64	1:16
	24-11	1:32	1:16	1:16	1:256	1:32	1:8	1:256	1:64	1:8
	12-2	<1:2	1:16	1:32	1:8	1:16	1:8	1:8	1:16	1:16
Bacterin	26-12	1:8	1:16	1:8	1:4	1:8	1:8	1:32	1:32	1:8
	5-6	1:8	1:8	1:8	1:4	1:8	1:4	1:4	1:8	1:4
	17-13	1:2	1:8	1:16	1:4	1:8	1:16	1:4	1:8	1:16
Control	30-6	<1:2	1:8	1:16	<1:2	1:4	1:16	<1:2	1:4	1:16
	19-8	1:8	1:16	1:8	1:8	1:64	1:8	1:8	1:64	1:8
	30-1	1:4	1:16	1:16	1:4	1:16	1:16	1:4	1:16	1:16

first and second injection to K99 pili antigen. No consistent change in titer could be detected in any group in response to 987 antigen. In reference to the bacterin group two of the gilts demonstrated a two-fold rise in titer to K88 antigen and one showed a decline in titer. This same group had only one gilt that responded with a two-fold rise in titer to K99 antigen while the other two gilts did not respond or show any change at all. In the Control group only one titer changed extensively, a four-fold rise in titer to K99 in one of the gilts. The Crude pili group showed consistently better response in a four-fold rise in titer to K88 and K99 while the Bactrin group only showed a two-fold rise at best to these antigens. In comparison, the Control group showed little to no response to K88 and the one gilt showed two-fold rise to K99 while the other two demonstrated no rise in titer to the K99 antigen.

The statistical analysis of the serum titers of all the sows in the study showed significant correlation between the strain of pilated organism given, and the rise in titers seen. This was especially true of the K88 serum antibody response which was significant at a .0001 alpha level. Again relying on statistical means the response to the K99 antigen could not be shown to be significant at a .05 level, but was at a .1 level, an indication that the two-fold rise in titer seen was not as strong as the response to the K88 antigen. The 987 antibody titers were not high enough to be significant in statistical terms on the prevaccination and first injection samples, however there was a significant response (.001 level) on the second injection sample.

There was a significant correlation between type of vaccine given and the antibody response toward all three pili types for all serum samples taken except in one instance. This was the response to 987

organism after the second injection, which did not consistently rise or fall in any of the vaccinated or control groups.

There was a significant correlation between the serum antibody responses to K88 and K99 antigens, to the .001 alpha level. No correlation was seen between 987 and K88 that was statistically significant, and there was no correlation between the K99 and 987 titers even though they have the same "O" or somatic antigen.

Colostrum Titers

The colostrum samples were collected immediately postparturient and frozen. Titers were determined by plate agglutination and the results are recorded in Table 4.

Table 4. Colostral Titers-Immediately Post Parturient					
Vaccine Group	Dam No.	<u>Challenge Organisms</u>			
		K88ab	K99	987	K88ac
Pili	27-6	1:128	1:4	1:64	--
	24-11	1:64	1:8	1:2	1:16
	12-2	1:64	1:8	1:2	1:4
Bacterin	26-12	1:16	1:16	1:8	--
	5-6	1:64	1:4	1:4	--
	17-13	1:16	1:4	1:16	--
Control	30-6	1:32	1:4	1:2	--
	19-28	1:2	1:4	--	--
	30-1	1:32	--	1:16	--

The titers for the challenge groups in the colostrum data demonstrates the pili group to have roughly two-fold higher titers than the bacterin group to antigen K88_{ab} and K99. The 987 antigen does not show any definite pattern; the titers recorded in this study do not indicate any one type of antigen to be superior to no antigen at all. The K88_{ac} antibody response is only seen in the crude pili group even though the antigen was present in both the pili vaccine and the whole bacterin. The control group titers to K88_{ab} demonstrated in the gilts here to be double those in the bacterin group. The K99 titers are equal to those of the bacterin group and about one half those in the crude pili group.

Statistical analysis of "colostrum-type of vaccine" correlation, shows significance with respect to titers of K88, K99 and 987 antibodies in the colostrum, indicating a better response to the crude pili vaccine than to the whole bacterin, and of the two vaccines with respect to the control. This was confirmed by the significant "serum level-colostral level" correlation which held true for alpha at .01 levels.

Although no relationship was found between the serum levels and weight gained, there was a significant correlation between numbers of E. coli present and weight gained. There was also a significant correlation between colostrum titers and weight gained for the first four days after birth. No correlation could be seen between colostral titers and weight gained on day 5. There was also a significant difference in weight gained and challenge organism, with more weight gained by pigs challenged with 987 than with K99 or K88. The K88 challenged pigs gained the least, less than the K99 pigs.

DISCUSSION

The data obtained in this study would indicate passive protection is passed to suckling pigs of pregnant gilts vaccinated with crude pili. This protection refers to diarrheal disease caused by ETEC possessing homologous pilus types. Pigs suckling these gilts were more resistant clinically than those suckling control gilts or gilts vaccinated with whole killed organisms with identical pilus present. These results support the hypothesis that a crude pili preparation can be effective in conferring protection against ETEC infection.

The challenge pigs born to gilts vaccinated with the crude pili had lower incidence of diarrhea, diarrhea for shorter duration and greater weight gain from birth to day 5 of the test.

The data of the intestinal titers of challenge organisms/10 cm of ileum would indicate that the crude pili vaccine was superior in decreasing the numbers of ETEC present of pilus types K88 and 987. The number of organisms present of the K99 pilus type were equal in the bacterin and pilus challenge pigs. The control group had significantly higher numbers of organisms present than either the bacterin or the crude pili challenged pigs. As this study was not designed to determine the exact mechanism of protection, the assumption is based on correlation of high antibody titers versus weight gained, numbers of organisms present and clinical observations of diarrhea, correlated with necropsy and histopathological studies consistent with findings of colibacillosis in neonatal swine.

The circulating antibody titers in the pregnant gilts correlate with those done earlier in guinea pigs with K88 purified pili indicating the feasibility of the crude preparation of pili vaccine. These titers also

correlate well with those found in the colostral samples of this study for K88_{ab} and K99, where higher titers were found in the crude pili group than the bacterin group. The importance of these high serum and colostral titers is consistent with the proposed mechanism of pili function, that antibodies prevent bacterial colonization by inhibiting bacterial adhesion to the intestinal villus epithelial cells. Another mechanism is that these antibodies act in opsonizing or agglutinating the ETEC.

That the crude pili vaccine was not statistically superior to the whole bacterin may have been due to the small quantity of animals used in the test. The parameters used to compare the two vaccines as explained above would indicate that the crude pili is a superior means of protecting neonatal pigs from colibacillosis, without the risk of abortion due to endotoxin in the whole cell formalized vaccine.

REFERENCES

1. Morgan, R.L., R.E. Isaacson, H.W. Moon, C.C. Brinton, and C.C. To. 1978. Immunization of suckling pigs against enterotoxigenic *Escherichia coli* induced diarrheal disease by vaccinating dams with purified 987 or K99 pili: protection correlates with pilus homology of vaccine and challenge. *Infect Immun.* 22:771-777.
2. Isaacson, R.E., R.L. Morgan, H.W. Moon, and C.C. Brinton Jr. 1977. Immunization against enterotoxigenic *Escherichia coli* infected by vaccination with purified pili, p. 285-293. In *Proceedings of the Thirteenth Joint Conference on Cholera*. Publication no. (NIH) 78-1590. Dept. of HEW, Washington, D.C.
3. Nagy, B.H., W. Moon, R.E. Isaacson, C.C. To and C.C. Brinton. 1978. Immunization of suckling pigs against enteric enterotoxigenic *Escherichia coli* infection by vaccinating dams with purified pili. *Infect. Immun.* 21:269-274.
4. Hohmann, A., and M.R. Wilson. 1975. Adherence of Enteropathogenic *Escherichia coli* to Intestinal Epithelium in vivo. *Infect. Immun.* 12:866-880.
5. Arbuthnott, J.P., and C.J. Smyth. 1979. Bacterial Adhesion in Host/Pathogen Interactions in Animals. p. 165-198. In D.C. Ellwood, J. Melling and P. Rutter (ed.) *Adhesion of Microorganisms to Surfaces*. Academic Press, London.
6. Duguid, J.P., and D.C. Old. 1980. Adhesive Properties of Enterobacteriaceae, p. 185-217. In E.H. Beachey (Ed.) *Bacterial Adherence: Receptors and Recognition*. Series B Vol. 6. Chapman and Hall, London.

7. Brinton, C.C. 1965. The structure, function synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transports in gram negative bacteria. Trans. N.Y. Acad. Sci. 27: 1003-1054.
8. Fusco, P.A. To, S. To and C. Brinton Jr. 1978. The purification and characterization of four types of E. coli pili and the specificity of E. coli pili for immunity, colonization and adhesion. p. 60-70. In C. Miller (ed.) XIII US-Japan Conference on Cholera, Atlanta, Ga. September 1977. National Institute of Health Bethesda, MA.
9. Sack, D.A., and R.B. Sack. 1974. Test for enterotoxigenic Escherichia coli using Y1 adrenal cells in miniculture. Infect. Immun. 11:334-336.
10. Dean, A.G., Y.C. Ching, R.G. Williams, and L.B. Harden. 1972. Test for Escherichia coli enterotoxin using Infant Mice: Application in a study of diarrhea in children in Honolulu. J. Infect. Dis. 125:407-411.
11. R.E. Isaacson. K99 Surface Antigen of Escherichia coli: Purification and partial Characterization; Infection and Immunity. Jan. 1977 p. 272-279.
12. Nagy, B., H.W. Moon, and R.E. Isaacson. Colonization of Porcine Intestine by Enterotoxigenic Escherichia coli: Selection of Piliated Forms In Vivo, Adhesion of Piliated Forms to Epithelial Cells in Vitro, and Incidence of a Pilus Antigen Among Porcine Enteropathogenic E. coli. Infect. and Immun. 1977. p. 344-352.
13. Guinee, P.A.M., J. Veldkamp, and W.H. Jansen. 1977. Improved Minca Medium for the Detection of K99 Antigen in Calf Enterotoxigenic Strains of Escherichia coli. Infect. Immun. 15:376-378.

13. Markwell, M.A.K., S.M. Haas, L.L. Bieder, and N.E. Tolbert. 1978. Modification of the Lowry Procedure to Simplify Protein Determination in Membrane and Lipoprotein samples. *Anal. Biochem.* 87:206-210.
14. McMichael, J.C. and J.T. Ou. 1979. Structure of Common pili from *Escherichia coli*. *J. Bacteriol.* 138:969-975.
15. Moon, H.W., R.E. Isaacson and J. Pohlenz. 1979. Mechanisms of Association of Enteropathogenic *Escherichia coli* with Intestinal Epithelium. *Am. J. Clin. Nutr.* 32:119-127.
16. Kohler, E.M. 1978. Results of 1976 Field Trials with Oral *Escherichia coli* Vaccination in Sows. *UM SAC* 73:352-356.
17. Moon, H.W., E.M. Kohler, and R.A. Schneider et. al.: 1980. Prevalence of Pilus Antigens, Enterotoxin Types, and Enteropathogenicity among K88 Negative Enterotoxigenic *Escherichia coli* From Neonatal Pigs. *Infect. Immun.* 27:222-230.

PILUS ANTIGEN INCIDENCE AND CONFERRED IMMUNITY OF
WHOLE BACTERINS AND PILUS VACCINES IN GUINEA PIGS
AND PREGNANT GILTS

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ABSTRACT PAPER I

Incidence of Pilus Antigens Associated with Enterotoxigenic Producing Escherichia coli Isolated By the KSU Veterinary Diagnostic Laboratory

Enterotoxigenic Escherichia coli isolated from pigs and calves were examined for the presence of K88, K99 or 987 pili. A total of 42% possessed K88 pili, 33% produced K99 pili and 3% produced 987 pili. The remaining 22% did not express any pilus production according to the plate agglutination test. The K88 E. coli were isolated primarily from swine and the K99 came from calves with severe diarrhea.

ABSTRACT PAPER II

A Comparison of Escherichia coli Bacterin Vaccine and Purified Pili Vaccine in Guinea Pigs

Escherichia coli serotype K87-K88ab was used to produce a whole formalized bacterin and a purified pili vaccine. The pili was purified by homogenization, ammonium sulfate precipitation, and DEAE-Sephadex column chromatography. The vaccine was injected into 4 groups of guinea pigs with and without aluminum hydroxide adjuvant. The antibody titers were determined twenty-one days after the second injection by serial dilutions.

The higher titers of circulating antibody in guinea pigs injected with purified pili vaccine and aluminum hydroxide, led to the conclusion that the pili was a superior means of stimulating antibody response.

ABSTRACT PAPER III

Vaccination with Crude Pili and Whole Cell Bacterins in Pregnant Gilts to Confer Immunity to Offspring: A Comparative Study

This study designed to compare vaccination of pregnant gilts with a crude pili and a whole cell bacterin indicates that greater passive protection is passed to suckling pigs vaccinated with crude pili. Pigs suckling these gilts were more resistant clinically than those vaccinated with the whole cell bacterin or the control animals. Incidence of diarrhea, duration of diarrhea for shorter duration and greater weight gain from birth to day 5 of the test were seen in offspring of gilts vaccinated with the crude pili.

Intestinal titers of challenge organisms/10 cm of ileum indicate that the crude pili was superior in decreasing the numbers of ETEC present of pilus types K88 and 987. Organisms of the K99 pilus type were equal in whole bacterin and crude pilus vaccination groups.

Circulating antibody titers in the pregnant gilts were higher in those vaccinated with the crude pili and correlate well with titers found in the colostral samples to K88 and K99, which were also higher than in bacterin vaccinated gilts. No correlation to 987 titers could be shown in either serum or colostral samples.

The crude pili vaccine could not be shown statistically to be superior to the whole bacterin, which may have been due to the small quantity of animals used in the study. However, it seems difficult to explain how the pili could be statistically superior to the whole cell bacterin when each parameter was examined and not statistically superior when all the parameters were compared collectively.