Response of Gnotobiotic Pigs to Escherichia coli

O. P. Miniats, L. Mitchell and D. A. Barnum*

SUMMARY

In a study of the response of gnotobiotic pigs to coliform infections, 45 one-week-old germfree pigs were divided into five groups and each group was inoculated orally with a different strain of Escherichia coli. Three of these were enteropathogenic swine strains, P307[08:K87(B), K88 a,b (L):H19]; P570 [0138:K81]; P568[0141:K85a,b(B), K88a,b(L) :H4], one was a virulent human strain, H224, [026:K60(B6)], and one was a non-enteropathogenic swine strain, P581[OX13:K68]. It was attempted to protect a portion of the pigs with orally administered specific antisera and sera from non-immunized specific pathogen-free (SPF) pigs. Observations were made on the clinical response, bacterial counts of feces and intestinal contents, gross pathological changes, distribution of the organisms in organs and serum hemagglutinin titers.

Infection with E. coli P307 resulted in diarrhea, dehydration and death, unless the pig was protected with specific antiserum. The pigs infected with E. coli P570 had a transient diarrhea but retained their appetites and recovered. Those infected with the other three strains remained healthy throughout. No circulating hemagglutinating antibody against the test strains of E. coli could be detected in any of the pigs seven days or earlier post-inoculation.

Relationship could not be established between the numbers of viable E. coli in the feces and the presence of clinical colibacillosis. Orally administered specific antiserum afforded protection against strain P307, but did not reduce the numbers of E. coli in the gut or alter their distribution in the internal organs. This suggested that the protective effect of specific antibody in the intestine was due to its action on a metabolite (enterotoxin) produced by E. coli P307 rather than the organism itself.

RÉSUMÉ

Au cours d'une étude sur la réponse de porcs gnotobiotiques à l'infection par les coliformes, on divisa 45 porcelets gnotobiotiques, âgés d'une semaine, en cinq groupes; chaque groupe reçut, par voie orale, une souche différente d'Escherichia coli. Trois de ces souches étaient les souches pathogènes pour le porc P307[08: K87(B), K88 a,b (L):H19]; P570[0138: K81]; P568[0141:K85 a,b(B), K88 a,b (L):H4], une autre était une souche humaine virulente, H224 [026:K60 (B6)]. La dernière était une souche porcine non-pathogène, P581[OX13: K68]. On essaya de protéger une partie des porcelets en leur administrant oralement des antisérums spécifiques et du sérum provenant de porcs "SPF" non-immunisés. On étudia la réponse clinique des animaux, les comptages bactériens de fèces et du contenu intestinal, les lésions macroscopiques, la distribution des microorganismes dans les organes et les titres d'hémaglutinine sériques.

L'infection par E. coli P 307 détermina de la diarrhée, une déshydratation et la mort, à moins que le porcelet ne fut protégé par des antisérums spécifiques. Les animaux infectés avec la souche P 570 manifestèrent une diarrhée passagère, mais gardèrent un bon appétit et se rétablirent. Ceux que l'on infecta avec les trois autres souches, demeurèrent en bonne santé. On ne réussit pas à détecter d'anticorps hémagglutinants contre les souches d'E. coli utilisées dans les sept jours qui suivirent l'ingestion.

Il fut impossible d'établir une relation entre le nombre d'E. coli viables dans les fèces et une colibacillose clinique. Les antisérums spécifiques administrés oralement donnèrent une protection contre la souche P 307; ils ne diminuèrent cependant pas le nombre d'E. coli dans l'intestin ni ne modifièrent leur distribution dans les organes. Ceci permet de croire que l'action protectrice des anticorps spécifiques dans l'intestin était causée par leur action sur l'entérotoxine métabolisée par E. coli P 307 plutôt que sur le microorganisme lui-même.

*Department of Clinical Studies (Miniats) and Department of Veterinary Microbiology and Immunology (Mitchell and Barnum), Ontario Veterinary College, University of Guelph, Guelph, Ontario.

Present address of L. Mitchell: Warner Lambert Research Institute of Canada Ltd., Sheridan Park, Ontario.

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INTRODUCTION

The assessment of enteropathogenicity of *Escherichia coli* for pigs has been based essentially on two criteria:

(a) The association of a given strain with clinical colibacillosis in the field (23) and

(b) The reaction of ligated intestinal segments of pigs to injection with the test strain (4, 16, 21).

Experimental reproduction of colibacillosis in conventionally reared pigs is not a reliable criterion of enteropathogenicity, as following oral administration, the desired strain frequently fails to establish in the gastrointestinal tract of the host (12, 17). Germfree pigs, on the other hand, have been shown to become readily infected with *E. coli* and to vary in their response to different strains (6, 8, 15). Therefore, they provide a suitable model for the study of colibacillosis.

This is a report of the response of gnotobiotic pigs to five different strains of *E. coli*. A comparison of the clinical response, gross pathological changes, bacterial counts of the feces and intestinal contents, distribution of the bacteria in internal organs and the effects of orally administered sera and serum antibody response are given.

MATERIALS AND METHODS

Experimental Animals

The experimental animals were 45 gnotobiotic pigs derived by hysterectomy and reared three per isolator in a germfree state, according to the methods described by Alexander *et al* (1). When the pigs were six days old, they were monitored for bacterial sterility, and on the following day they were inoculated with one of the five test strains of *E. coli*. Nine pigs were given each strain. To test the effects of sera, one pig in each isolator was treated with homologous *E. coli* OK antiserum, the second pig was given normal serum obtained from specific pathogen free (SPF) swine reared in isolation, and the third pig received no serum. Four ml of the serum was administered orally 30 minutes prior to inoculation of the pigs with *E. coli* and thereafter at eight hour intervals for three days. Blood samples for serology were collected from the anterior vena cava prior to exposure and where possible at termination of the experiment. Non-contaminated pigs of the same age were maintained in a separate isolator as negative controls.

The pigs were observed for clinical signs of collibacillosis, particularly vomition, inappetance, diarrhea and dehydration. Fecal samples for bacteriological studies were collected from each pig at two, four, six, eight, 24, 48 and 168 hours (seven days) post inoculation (p.i.). In the event of death, necropsies were carried out within two hours. The pigs that survived were killed seven days p.i. with an overdose of Nembutal[1]. The internal organs of these animals were examined for gross pathological changes and for presence of bacteria.

Bacteriological Procedures

Monitoring for Bacterial Sterility — Monitoring of the germfree pigs for bacteriological sterility was carried out 24 hours prior to administration of *E. coli* according to our routine procedure. Swabs were collected from the mouth and the rectum of two pigs in each isolator and another swab was taken from a control milk can which was left open in the isolator for the duration of the experiment. Immediately after removal from the isolator, each swab was plated on two blood agar plates and then placed into a tube of thioglycollate broth. One of the blood agar plates was incubated aerobically at 37°C for 48 hours, and the other plate was incubated anaerobically for 24 hours. If these samples were bacteriologically negative after 24 hours incubation, the experiment was begun.

The inoculated thioglycollate tubes were incubated at 37°C for two weeks and observed daily for turbidity. At the end of the incubation period the samples were replated onto each of two blood agar plates and incubated in the same manner as described previously. If bacterial growth was not observed in any of the samples tested (which was the case in all instances during this work), the animals were considered as having been bacteria free prior to inoculation with *E. coli*.

Preparation of the Inocula — The *E. coli* strains used in this work are presented in Table I. These strains, originally isolated from their respective hosts and stored on agar slants at room temperature, were transferred to blood agar plates and incubated at 37°C for 24 hours. Organisms from

[1]Abbott Laboratories Limited, Montreal, Canada.

TABLE I. Escherichia coli Strains Used for Infecting Gnotobiotic Pigs

Strain Number	International Classification	Hemolysis	Source
P307	08:K87(B)K88 a,b(L):H19	+	Coliform enteritis in swine
P570	0138:K81	+	Coliform enteritis in swine
P568	0141:K85 a,b(B), K88 a,b(L):H4	+	Coliform enteritis in swine
H224	026:K60(B6)	+	Infantile diarrhea
P581	0X13:K68	−	Healthy swine

a smooth colony of each strain were inoculated into a different tube of tryptic soy broth. Following incubation for four to five hours at 37°C, these were placed into the entry port, each of a different isolator, and sprayed with two per cent peracetic acid where they remained overnight at 27°C to 30°C. Then the cultures were introduced into the isolators and diluted by the double dilution method to contain approximately 10^4 viable organisms per ml. One ml of the suspension was added to the morning meal of each pig. The remainder of the inoculum was removed from the isolator and used for the determination of bacterial counts.

The sera were prepared and sterilized as described previously (15). The specific agglutinin titers are presented in Table II.

TABLE[1] II. Agglutinin Titers[a] of Escherichia coli Antisera

Type of Serum	"O" Titer	"K" Titer
Anti P307.....	256	128
Anti P570.....	1024	128
Anti P568.....	512	32
Anti H224.....	1024	256
Anti P581.....	2048	64
Normal SPF pig...	0	0

[a]Expressed as reciprocals of serum dilutions.

Neither the antisera nor the normal pig serum caused agglutination of any of the heterologous *E. coli* antigens used in this study, as determined by the serial tube agglutination test, and the indirect hemagglutination test.

Examinations of Feces and Intestinal Contents — Feces were collected from the rectum of each pig with a sterile swab into a 16 ml tube. The two and four hour samples were streaked onto blood agar plates and onto McConkey's agar to determine if the organisms had reached the rectum. The six, eight, 24, 48 and 168 hour fecal samples and contents of the duodenum and colon collected during necropsy were used for counts of viable *E. coli* by modification of the Miles and Misra technique (13).

The samples were weighed in a preweighed plastic petri plate, suspended to 1/10 weight per volume in sterile normal saline and then diluted out to 10^{-8} volume per volume. Two 0.2 ml amounts of each dilution were placed on each of two McConkey agar plates and spread, giving four counts per dilution. After 12 to 16 hour incubation at 37°C, the number of colonies on each plate were counted and the number of viable *E. coli* per gm of sample calculated.

Serology — The somatic and capsular antibody titers of the sera were determined by the bacterial tube agglutination test following the method adopted from Edwards and Ewing (3) and described by Miniats (14). The indirect hemagglutination test method employing sheep red blood cells was adopted from those described by Sharpe (19) and Sojka (23).

Necropsy Techniques — Since the pigs had heavy body surface contamination with the test strains of *E. coli* aseptic necropsy techniques were employed to avoid contamination of the internal organs. Following death, the animal was placed on a sterile tray with the left side uppermost. The skin was removed from the left side and folded back ventrally and dorsally. Then the entire pig was wrapped tightly in sterilized aluminum foil. The thoracic and abdominal cavities were exposed by removing with sterile instruments, the foil covering and the skinned part of the body wall. The brain, heart, kidney, mesenteric lymph nodes, portions of the lung, liver and spleen, a section of the small intestine 30 cm posterior to the stomach and a section of the large intestine were removed and placed into individual sterile petri plates. The surface of each organ was seared with a

TABLE III. Clinical Response of Gnotobiotic Pigs During Three Days Following Inoculation with Escherichia coli

E. coli Strain	Serum Treatment[a]	No. Pigs Used	No. Pigs Sick	No. Pigs Died	Onset of Scours P.I.[b]	Time of Death P.I.	Intest. Lesions	Remarks
P307	None	3	3	2	18 hrs.	32 hrs.	2	Severe diarrhea
	Normal	3	3	3	16 hrs.	33 hrs.	3	Severe diarrhea
	Specific	3	1	0	24 hrs.	—	1	One pig — transient diarrhea
P570	None	3	3	0	24 hrs.	—	0	All had diarrhea which subsided in 72 hours
	Normal	3	3	0	24 hrs.	—	0	
	Specific	3	3	0	24 hrs.	—	0	
P568	None	3	0	0	—	—	0	All normal
	Normal	3	0	0	—	—	0	All normal
	Specific	3	1	1	—	18 hrs.	0	One pig — peritonitis ruptured intestine
H224	None	3	0	0	—	—	0	One pig — transient inappetance
	Normal	3	0	0	—	—	0	One pig — transient inappetance
	Specific	3	0	0	—	—	0	All normal
P581	None	3	0	0	—	—	0	All normal
	Normal	3	0	0	—	—	0	All normal
	Specific	3	0	0	—	—	0	All normal

[a]The sera were administered orally.
[b]P.I. = Post Inoculation.

heated spatula and the samples for bacteriological examination were collected through an incision made with a sterile blade in the seared part.

RESULTS

Clinical Response and Necropsy Observations

A summary of the clinical response of gnotobiotic pigs to inoculation with each of the five strains of *E. coli* is presented in Table III. Five of the six pigs given *E. coli* P307 (a strain commonly isolated from severe or fatal cases of baby pig diarrhea) and given no serum or normal SPF pig serum, exhibited a severe clinical response manifested by vomition, inappetance, diarrhea, rapid dehydration and death. The diarrhea started eight to 24 hours (average 20 hours) p.i., and the deaths occurred in 15 to 50 hours (average 33 hours).

Apart from one pig which had a temporary diarrhea, the pigs given specific P307 antiserum remained healthy while the serum was administered but two of them died two days following withdrawal of the serum. One of the pigs given no serum and one treated with specific antiserum survived and appeared healthy until the termination of the experiment at seven days.

All of the nine pigs infected with strain P570, which is commonly isolated from swine affected with post weaning enteritis, had a transient diarrhea 24 to 72 hours p.i. regardless of whether they were treated with specific antiserum or not. They exhibited no other clinical signs and after recovery from diarrhea, were comparable to the negative controls. The pigs given the other three strains remained essentially normal throughout the experiment.

Grossly visible pathological lesions were observed only in those pigs which died as a result of infection with *E. coli* P307. Some of these animals had congested lymph nodes, and all of them had congested areas in the small intestine with dilated segments that were filled with gas, mucus-containing fluid and incompletely digested milk. None of the other pigs had such lesions.

Bacteriological Observations

E. coli were first isolated from the feces of nine of the forty five gnotobiotic pigs six hours p.i. and of the remaining thirty six pigs in eight hours. The average num-

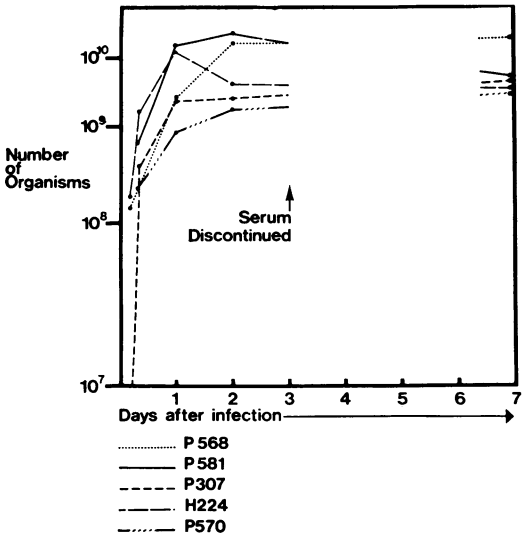

Fig. 1. Number of viable organisms per gm of feces of gnotobiotic pigs, monocontaminated with Escherichia coli as related to time after inoculation. Data from serum treated and non treated animals is combined.

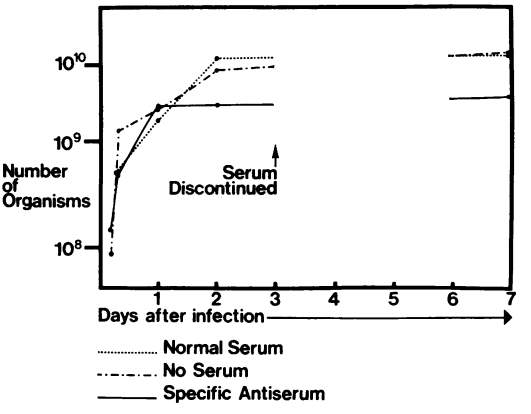

Fig. 2. Effect of orally administered sera on Escherichia coli counts in the feces of monocontaminated gnotobiotic pigs expressed as number per gm of feces. Data from pigs inoculated with five strains of E. coli is combined.

ber of viable *E. coli* per gram of feces disregarding serum treatment as related to individual strains of *E. coli* and time after inoculation, is presented in Fig. 1.

The organisms increased in numbers rapidly during the first 24 hours and in some instances during the second day. Eight hours p.i. the counts ranged from 10^5 to 10^8 of viable *E. coli* per gm of feces. The 24 and 48 hour samples collected from pigs with *E. coli* strains P307, P570 and H224 had 10^8 to 10^9 organisms per gm of feces, while those with *E. coli* strains P568 and H224 had counts in the 10^{10} range. After the first 48 hours, the levels remained relatively constant. The *E. coli* counts of the contents of the duodenum following natural death or when the pigs were killed seven days p.i. were in the range of 10^7 to 10^8 in the case of strains P307, P570 and H224, and 10^9 in the case of the strains P568 and P581. The number of organisms was about ten times higher per gm of contents in the large intestine than in the small intestine (Table IV). The *E. coli* counts in feces were on the average, about one log lower in the pigs treated with specific antiserum, than in those not so treated (Fig. 2). However, in individual cases, the counts were frequently as high or even higher in the pigs that received specific antiserum than in the animals that received no serum or normal SPF pig serum. Fig. 3

TABLE IV. Viable Escherichia coli in Intestinal Contents of Monocontaminated Gnotobiotic Pigs After Natural Death or Seven Days Following Inoculation[a]

Strain of E. coli	Time p.i.	Duodenum	Colon
P307	Died in. 15-50 hrs.	1.0×10^8	4.2×10^8
P307	Killed in 7 days	6.1×10^8	4.8×10^9
P570	"	1.5×10^7	4.4×10^9
P568	"	1.2×10^8	2.7×10^{10}
H224	"	5.6×10^8	5.0×10^9
P581	"	5.1×10^8	7.0×10^9

[a]Expressed as number of organisms per gram of contents.

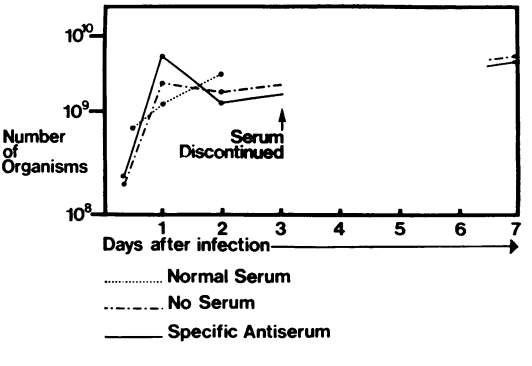

Fig. 3. Effect of orally administered normal SPF pig serum and specific Escherichia coli G7 antiserum on counts of the homologous strain of Escherichia coli, expressed as number per gm of feces.

TABLE V. Distribution of Escherichia coli in Internal Organs of Monocontaminated Gnotobiotic Pigs

E. coli Strain	Serum Treatment	No. Pigs Exam	Time of Death	Number of Pigs with Infected Organs: Heart's Blood	Lung	Liver	Spleen	Kidney	Brain	Mes. Lymph Node	Stomach	Duodenum	Colon
P307	None	2	Died in 32-33 hours	2	2	1	1	1	2	2	2	2	2
		1	Killed 7 days p.i.	0	0	0	0	0	0	1	1	1	1
	Spec. Orally	2	Died in 24-48 hours after last treatment	1	0	0	1	1	0	0	2	2	2
		1	Killed 7 days p.i.	0	0	0	0	0	0	1	1	1	1
	Non Spec.	2	Died in 15-50 hours p. i.	1	0	0	1	2	1	2	2	2	2
	Combined[a]	8		4	2	1	3	4	3	6	8	8	8
P570	Combined[a]	6	Killed 7 days p.i.	1	0	0	1	1	3	5	6	6	6
P568	Combined[a]	8	Killed 7 days p.i.	1	0	0	2	2	4	4	8	8	8
H224	Combined[a]	6	Killed 7 days p.i.	1	1	1	1	1	2	3	4	6	6
P581	Combined[a]	7	Killed 7 days p.i.	0	0	0	0	0	2	3	7	7	7
TOTAL	Combined[a]	35		7	3	2	7	8	14	21	33	35	35

E. coli isolations from organs regardless of serum treatment.

shows that in the case of strain P307, where oral administration of the homologous antiserum was demonstrated to have a protective effect, the bacterial counts did not differ significantly between the treated and non treated pigs.

The distribution of the five strains of *E. coli* in the internal organs of the experimental gnotobiotic pigs is presented in Table V. The respective strains of *E. coli* were isolated from the small and large intestines of all of the pigs, but only from 33 of the 35 stomachs examined. The next organs most frequently yielding the organisms were the mesenteric lymph nodes (21/35) and the brain (14/35). The virulent strain P307 was the most invasive and the non-virulent swine strain P581 was the least invasive, as the latter strain could be isolated consistently only from the digestive tract, and occasionally from the mesenteric lymph nodes and the brain. Oral administration of specific *E. coli* antiserum did not influence the distribution of the organisms in the internal organs.

Serology — None of the pigs had detectable serum hemagglutinating antibody against the test strains of *E. coli* seven days or earlier following inoculation.

DISCUSSION

In this study, gnotobiotic pigs were exposed to no other known microflora than the *E. coli* strain used for inoculation of each group. Since all other conditions in the environment of the animals were comparable, the differences in their response must have been related to the *E. coli* strain variations. Differences in the virulence of *E. coli* to a given species have been attributed to the colonizing ability and enterotoxin production of the different strains (5, 20, 21, 23). In this work prime attention was given to the colonizing abil-

ity of the organisms as determined by their numbers in the feces soon after inoculation, and in the feces and small intestine at termination of the experiment.

The strains P307 and P570 proved to be virulent even though their numerical values did not exceed those of the strains that did not cause diarrhea. In addition to the above two strains, *E. coli* P568 also has been demonstrated to be enteropathogenic by the intestinal loop model (4, 16, 21) and in conventional pigs (2, 17, 18). In the present study, this strain reached higher numbers than the two former strains, but failed to cause diarrhea. Thus variation in numbers of organisms was not related to enteropathogenicity. These findings appear to disagree with those of Smith and Jones (20) who reported a direct relationship between the numbers of enteropathogenic *E. coli* in the upper small intestine and colibacillosis in conventionally reared pigs, but are in accord with those of Kohler (8) who could not establish such a relationship in orally inoculated gnotobiotic pigs. Two factors may account for the difference between our findings and those reported in conventional animals. It is possible that under conditions where other competing organisms are present, virulent strains of *E. coli* have a greater potential at proliferation in the small intestine than non-virulent strains do. Secondly, to allow sufficient time for clinical observations, most of our pigs were killed and bacterial counts of the contents of the small intestine carried out on the seventh day p.i. It is possible that fluctuations in the numbers of *E. coli* that may have occurred there earlier were not reflected in the counts of the fecal contents. Studies related to these problems are in progress. The failure of *E. coli* P568 to cause diarrhea could be explained by it having lost its virulence during storage and subculture in the laboratory (16, 17) or it being a non-enteropathogenic mutant of the same serotype (16).

The strain H224, virulent to human infants, established equally well in gnotobiotic pigs as did the swine strains, but failed to cause disease. This result, which correlates with the negative ligated pigs intestine test of the same strain (4), denotes host specificity.

The protection given by orally administered specific antiserum against *E. coli* P307 is in agreement with that reported by Kohler (7, 9). In our work this effect too could not be attributed to the reduction of the number of the organisms.

Since enteropathogenic and non-enteropathogenic strains of *E. coli* appeared to have equal potential at proliferation in the alimentary tract of gnotobiotic pigs, the ability of a given strain to cause diarrhea must have been dependent upon other factors which, at least in the case of *E. coli* P307, were neutralized by orally administered specific antiserum. Such factors (enterotoxins) have been demonstrated to be present in cell free culture fluids (5, 10, 11, 22) and in whole cell lysates of enteropathogenic *E. coli* (5). The enterotoxins obtained by different methods differ somewhat in their physical and chemical properties, but their physiological activity is similar; they cause positive gut loop reactions (5, 22) and transient diarrhea in conventional and gnotobiotic pigs (10, 11) in the absence of viable enteropathogenic *E. coli*. It has also been shown that the enterotoxic activity of the whole cell lysate may be neutralized by the homologous and in some instances heterologous enteropathogenic *E. coli* antiserum (5). The findings of this work would thus be in agreement with the contention that the pathogenesis of coliform enteritis in pigs is related to enterotoxin production by certain strains of *E. coli*. It is likely that a certain minimal number has to be reached by these strains in the small intestine in order to produce sufficient enterotoxin for diarrhea to occur. However, in the case of *E. coli* P570, the reaction of the host did not fit the usual pattern. In our work, this strain caused diarrhea which could not be prevented by the administration of specific antiserum. Gyles (5) found that the same strain produced strong reaction when it was tested in a ligated intestinal loop as a whole culture, while the whole cell lysate of this strain failed to produce positive reactions.

It appears that marked differences exist among the enteropathogenic strains of *E. coli* and that enteropathogenicity may not be a constant property even of a given serotype or strain.

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