COMPARISON OF ESCHERICHIA COLI VIRULENCE FACTORS WITH COLONY MORPHOLOGY ON VARIOUS MEDIA

by

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INTRODUCTION

Escherichia coli is widely distributed in nature as a normal inhabitant of the intestinal tract of man and animals. It is excreted in great numbers in feces; and serves as an indicator of fecal contamination of the environment.

Certain strains of E. coli are pathogenic for man and animals. Acute infantial diarrhea, traveler's diarrhea and colibacillosis of newborn calves, pigs and lambs have been associated with enteropathogenic strains. Even strains considered non-pathogenic normally found in the intestine appear to be opportunistic pathogens, causing peritonitis following abdominal operations, rupture of the intestine and wounds perforating the digestive tract (Bruner and Gillespie, 1973). It has also been found as a secondary invader in many pathological conditions (Merchant and Packer, 1967).

Pathogenesis of enterotoxigenic E. coli (ETEC) mediated diarrhea has been shown to be dependent upon the expression of two virulence factors. These factors are; the elaboration of antigenic surface pili that promote bacterial colonization of the small intestine; and the production of enterotoxin (Isaacson et al., 1978a). Although these factors can be detected by various in vivo and in vitro systems, there is need of a more reliable, rapid and simple assay to detect enteropathogenic E. coli (Sack et al., 1975; Dean et al., 1972; and Isaacson et al., 1975).

The relationship between these virulence factors and the colony morphology on various media has not been studied. In the present

investigation, the colony morphology of E. coli on various media and their correlation with enterotoxin production and/or presence or absence of pili was studied in an attempt to devise and easily conducted technique for detection.

REVIEW OF THE LITERATURE

Enteric disease of newborn animals caused great economic loss to the livestock industry (Wohlgemuth, 1977; House, 1978; and Söderlind et al., 1976). Most figures concerning morbidity and mortality were based on estimations because accurate and specific etiologic diagnosis was difficult to obtain, due to the multiplicity of infectious agents involved, the apparent non-specificity of signs and lesions associated with some agents and the frequent occurrence of subclinical infections (Acres et al., 1975; and Fey, 1972). House (1978) estimated the loss to the livestock industry from enteric disease of calves at approximately $95.5 million per year over a 7 year period (1970-1976). Fifty and nine tenths to 74.6 percent of the loss ($48.6 - 71.2 million) was due to infection in which enteropathogenic E. coli were involved. Mayer et al. (1964) reported an annual loss of 5-26% of the calves in West Germany which resulted in an economic loss of 57 million D.M. Amstutz (1965) estimated the death loss at 8-25% with an economic loss of $42 million per year. Similar losses were reported in Denmark (Ottosen, 1959). Stevens (1963a and 1963b) estimated that nearly 75 percent of all baby pig diarrhea was associated with E. coli infection, but did not estimate monetary loss.

Escherichia coli mediated neonatal diarrhea was discovered to depend on two major attributes of microbial virulence, the ability to produce enterotoxin (Smith and Halls, 1967; and Gyles and Barnum, 1969) and the ability to attach to cells and colonize the small intestine (Jones and Rutter, 1972; and Burrows et al., 1976). Strains of E. coli which

possessed these virulence factors were termed enterotoxigenic (ETEC).

Colonization of the small intestine by ETEC depended upon the ability of the microorganisms to multiply and resist peristaltic activity. This was accomplished by adhesion of the ETEC to intestinal epithelial cells (Nagy et al., 1976). Adhesion was found to be mediated by antigenic, protein, hair-like appendages on the surface of the bacterial cells termed pili (Stirm et al., 1967 a and 1967b).

Kauffman established the modern diagnostic antigenic scheme for E. coli. He discovered that the thermostable somatic (O) antigen was masked by a thermolabile capsular (L) antigen which rendered living strains O-inagglutinable. With collaborators he found two additional heat-labile superficial antigens designated A and B. These three antigens were grouped under capsular (K) antigens with the sub-types A, L and B. They formed either true capsules (A) or envelopes (L and B). They were differentiated on the basis of heat stability and agglutinin-binding capacity (Edwards and Ewing, 1972). Ørskov et al. (1977) expressed the opinion that in light of recent studies the classic K-A, L, and B antigenic scheme of Kauffman was outdated. He quoted unpublished data which indicated that at least some K antigens were lipopolysaccharide in nature. Strains which possessed K antigen were more toxic, resistant to phagocytosis, and the bactericidal action of antibody (Kauffman, 1954; and Sojka, 1965).

It appeared that strains which possessed certain O and K antigens were more important in diarrheal disease. Serotype O 137 played a major role in England (Rees, 1958) and O 101 in Ontario and the United States (Gossling et al., 1964; Glantz et al., 1969; and Glantz, 1971). Although

serological identification of O antigens of E. coli was valuable in epidemiological studies, healthy animals often harbored strains of the same serotype.

The K88 antigen was first described by Ørskov et al. (1964) on ETEC from swine. It was an L type of K antigen, destroyed at 100 C, and not formed when cells were grown at 18 C. The antigen existed in two serologically different forms, K88ab and K88ac. Stirm et al. (1967a and 1967b) demonstrated that the K88 antigen formed a layer of fine filaments on the surface of the cell which were pili or pili-like. Ørskov and Ørskov (1966) demonstrated that K88 synthesis was controlled by extrachromosomal DNA which was episomal or plasmid in nature. Strains carrying the trait spontaneously lost it and were able to transfer the characteristic by conjugation. Treatment of K88 positive ($K88^+$) strains with ethidium bromide also resulted in loss of the trait (Jones and Rutter, 1972). Guinée and Jansen (1979) described a third form, K88ad, by hemagglutination, immunoelectrophoresis and double diffusion techniques. The various K88 antigens differed in their electro-endosomotic flow, which indicated a difference in structure. Strains of E. coli which possessed the K88 antigen attached to cells in vivo and in vitro. This ability was considered the reason why they overcame intestinal motility and proliferated rapidly in the lumen (Wilson and Hohman, 1974; Jones and Rutter, 1972; and Bertschinger et al., 1972). Wilson and Hohman (1974) demonstrated that adhesiveness of K88 was associated with the c and b antigens and that this characteristic was neutralizable only by homologous antisera. Jones and Rutter (1972) studied the role of K88 in the pathogenesis of neonatal diarrhea in pigs with $K88^+$ and

K88 negative ($K88^-$) strains. The $K88^-$ strains were derived from the $K88^+$ strains, but had lost the plasmid for K88 production spontaneously or by ethidium bromide treatment. The $K88^+$ strains synthesized the antigen in the intestinal lumen, adhered to the mucosa and killed 50% of conventionally reared piglets. The $K88^-$ strains failed to adhere or colonize and killed only 3% of the pigs. They concluded that the K88 antigen was responsible for attachment of $K88^+$ E. coli to the wall of the small intestine and that adhesion was essential for virulence.

An additional K antigen of E. coli which was enteropathogenic for calves and lambs was described by Smith and Linggood (1971b). This antigen, originally termed Kco, was designated K99 by Ørskov et al. (1975). The K99 antigen resembled K88 in many respects, it was shared by E. coli strains in several different O groups, was not synthesized at 18 C, was thermolabile, protein in nature, and had a filamentous structure. Isolates possessing K99 antigen adhered to intestinal epithelium, colonized the intestinal lumen, and produced diarrhea (Jones and Rutter, 1974; Burrows et al., 1976; and Guinée et al., 1976). Studies similar to those conducted with K88 in pigs established that K99 was also an important virulence factor for ETEC in calves (Smith and Lingood, 1971b; Isaacson et al., 1978a; and Isaacson, 1977). Moon et al. (1977) recovered K99 positive ($K99^+$) strains of E. coli from pigs with diarrhea. Pigs inoculated with these strains reacted similarly to those exposed to $K88^+$ strains.

Nagy et al. (1976) studied the ability of several $K88^-$ enteropathogenic E. coli to colonize the small intestine of the pig. They concluded that some $K88^-$ EEC did colonize the ileum and produced diarrhea in

newborn pigs. The preference for the ileum differed from K88$^+$ strains in that they colonized both the ileum and jejunum (Arbuckle, 1970 and Jones and Rutter, 1972). One of these strains, 987, was studied further and demonstrated ability to adhere to intestinal mucosa by means of a pilus (Nagy et al., 1977; and Isaacson et al., 1978b). This pilus belonged to the general class of somatic pili, was composed of rod like assemblies of a single kind of protein sub-unit, and was serologically distinct from K88 and K99 (Morgan et al., 1978). Isaacson et al. (1978a) recovered 5 isolates of E. coli from calves which possessed 987 P antigen, but they were not apparently associated with diarrhea.

Since the virulence factors K88, K99 and 987 P were demonstrated frequently in ETEC of animal origin, they were considered significant diagnostic features (Isaacson et al., 1978a; Jones and Rutter, 1972; and Moon et al., 1976). Slide agglutination (Guinée et al., 1976), immunoelectrophoresis (Cahill and Glantz, 1978; Guinée et al., 1977a; and Guinée et al., 1979), immunofluorescent staining (Moon et al., 1977), and hemaglutination (Jones and Rutter, 1974, Guinée et al., 1979; and Burrows et al., 1976) have been utilized. However, these techniques were time consuming and required up to 4-5 days with some strains.

Ørskov et al. (1975) reported that the K99 antigen was more easily detected if transparent colonies were selected. This observation was confirmed by Moon et al. (1976). When opaque colonies were selected, 5 of 60 strains were K99$^+$, but when translucent colonies were selected from the same strains 28 of 60 were positive. Guinée et al. (1977b) recognized the difficulty of demonstration of the K99 antigen. They observed that the antigen was not detected in some ETEC when grown on

common laboratory media due to excess capsule formation. They devised a minimal casein medium (Minca) which aided in expression of the antigen. They further improved the medium by the addition of IsoVitalex* (Improved Minca medium). No difference in colony morphology was noted. Isaacson et al. (1978a) investigated several procedures to improve detectability of K99. They found that passage of cultures each day for 4 days in Trypticase Soy Broth* with vigorous shaking, followed by plating on Improved Minca agar improved detectability of the antigen.

Pili were also recognized as virulence factor of other infectious agents. Four distinct morphologic colony types (1, 2, 3 and 4) of Neisseria gonorrhoea were recognized with the aid of a dissecting microscope and associated with virulence (Kellogg et al., 1963). Types 1 and 2 were virulent and types 3 and 4 nonvirulent. The colony types were later associated with the presence or absence of pili on the cells. Types 1 and 2 were piliated and types 3 and 4 nonpiliated (McGee et al., 1977).

The production of enterotoxin by ETEC was first described by Smith and Halls (1967). This toxin was heat-stable (ST), dialyzable and non-immunogenic. A second E. coli enterotoxin which was heat-labile (LT) was described two years later (Gyles and Barnum, 1969). This toxin was nondialyzable and immunogenic. Following colonization, ETEC strains produced one or both of these toxins which induced excess isotonic fluid secretion into the intestinal lumen with subsequent expulsion of liquid

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*BioQuest, Cockeysville, Maryland.

feces. Sustained fluid and electrolyte loss caused dehydration, hemoconcentration, acidosis and often led to death (Kohler, 1972). Escherichia coli of pigs produced LT, ST or both (LT/ST). All three of these types were associated with clinical diarrhea (Smith and Gyles, 1970a). Calf ETEC produced either ST or LT but not both (Smith and Halls, 1967; and Smith and Gyles, 1970b).

The biological activities of LT and ST were established using different assay systems. These assays were the production of ligated intestinal loops in rabbits, pigs or calves for detection of LT or ST (Moon et al., 1970a; Evans et al., 1973; Guinée et al., 1977a; and Myers et al., 1975), a cell culture assay with Y-1 adrenal cells for detection of LT(Sack and Sack, 1975 and Donta et al., 1974) and an infant mouse inoculation for detection of ST (Dean et al., 1972 and Giannella, 1976). Additionally two serologic procedures, solid-phase radioimmunoassay (Ceska et al., 1978) and passive immune hemolysis (Evans and Evans, 1977) were developed for the detection of LT.

The LT resembled Vibrio cholera enterotoxin in biological activity. Both LT and CT were antigenically similar (Holmgren et al., 1973 and Gyles, 1974a), and stimulated adenylate cyclase activity in Chinese hamster ovary (CHO), mouse adrenal cortex tumor (Y-1), and African green monkey (Vero) cultured cell lines (Guerrant et al., 1974; Donta et al., 1974; Sack and Sack, 1975; and Stavric et al., 1978). Morphologic changes and an increase in intracellular cyclic adenosine monophosphate (cAMP) were noted in LT treated cells. The Y-1 adrenal cells additionally produced Δ^4, 3 ketosteroid. An increased level of adenylate cyclase activity was also present in intestinal mucosal cells in vivo (Gorbach

and Khurna, 1972 and Kantor et al., 1974). This increase was considered the pathogenic mechanism by which LT caused the influx of fluid into the intestinal lumen.

Mundell et al. (1976) studied factors which influenced LT activity using Y-1 adrenal cells. They found that an enriched medium containing 0.6% yeast extract, 2% casamino acids, and 0.2% glucose buffered at pH 8.5 produced the highest LT activity. Activity was markedly decreased if the initial pH of the culture media was reduced to pH 7.5 or less. Activity of LT was also reduced by one-third when culture filtrates were heated at 60 C for 5 minutes. More than 90% of the steroidogenic activity was lost after heating at 60 C for 15 minutes. Crude-culture filtrates containing LT were stored at 4 C for several days without appreciable loss of activity. Lyophilization or freezing at-70 C was recommended for long term storage. Recently Kunkel and Robertson (1979) supported the previous findings that LT activity increased when the pH of the medium was above 7. The release of cell-associated LT was significantly reduced by preincubation with protease inhibitors and increased by pre-incubation with trypsin. They also found three molecular weight species of LT by using gel electrophoresis of polymyxin B extracts. The 22,000 and 30,000 dalton fragments were active in the pigeon erythrocyte lysate assay (PEL). The 72,000 dalton fragment was active in both the PEL and Y-1 adrenal tumor cell assay. They implied that the 72,000 dalton fragment was the holotoxin of LT. Donta et al. (1974) studied the effect of culture filtrates of both ST and LT producing strains of ETEC in Y-1 adrenal cells. The cells failed to elicit a morphologic response when treated with ST. They suggested that the cells lacked appropriate

receptors for ST.

Dean et al. (1972) described an infant mouse assay for biologic activity of ST based on its ability to produce fluid movement from the body into the intestinal lumen. Following intragastric inoculation and incubation of suckling mice, they were euthanitized, the entire intestinal tract removed and the ratio of gut weight to remaining carcass weight calculated. Giannella (1976) refined the conditions under which the assay should be conducted. Jacks and Wu (1974) studied the biologic and biochemic properties of ST in infant mice and found that it caused fluid accumulation within 2-4 hours. Heat-labile toxin did not react in infant mice even when incubated for 8 hours. They purified ST using ultrafiltration and Sephadex-200. This preparation contained 15% protein and 2% carbohydrate, was resistant to acid, trypsin and pronase, and its activity was lost after heat treatment at 100 C for 30 minutes.

The possibility that ST existed in two forms was suggested by the work of Moon and Whipp (1970b). They classified ETEC into two distinct groups. Class I strains produced toxin active in pigs of all ages but class II strains were active only in piglets. Burgess et al. (1978) classified ST into 2 different forms based on biological activity. The two forms were separated by methanol extraction into STa and STb. Stable toxin a was methanol soluble, partially heat stable, active in 1-3 day old neonatal piglets and infant mice, but inactive in 7-9 week old weaned pigs. Stable toxin b was methanol insoluble, active in weaned pigs and rabbit ligated intestinal loops, but inactive in infant mice. They concluded that the infant mouse assay failed to identify certain ST producing strains and recommended that the assay for ST should not

be based solely on the infant mouse assay. The rabbit ligated loop assay should be conducted on those suspect strains which were negative to the infant mouse assay.

Myers and Guinée (1976) surveyed 1004 isolates of E. coli, that were collected from calves with diarrhea during the spring of 1975. One hundred twenty four isolates were enterotoxigenic based upon their ability to cause distention of calf ligated intestinal segments. One hundred and fourteen of the 124 isolates of ETEC were placed in to six different groups by agglutination in OK antiserum. One hundred twenty non-ETEC were also agglutinated by OK antiserum of the same groups. Serogroups were O9:K35, O101:K30, O8:K85, O20:K?, O8:K25 and O101:K28. The K99 antigen was detected on 28 of 35 isolates of ETEC within these serogroups. It was not detected on any of 10 isolates of non-ETEC. Recently Isaacson et al. (1978a) examined calf ETEC and found that all clinically important isolates which were enterotoxin producers were also $K99^+$. These strains fell into 7 different biotypes according to carbohydrate fermentation and amino acid decarboxylation tests. However, not all isolates in these biotypes were ETEC. Moon et al. (1976) studied the etiology of diarrheal disease of calves in Minnesota and Montana and found that ETEC was involved in about 10% of the calves. Forty-six of 345 isolates studied were ETEC. Thirty-five of the 46 isolates were $K99^+$. Only 9 of 66 non-ETEC were $K99^+$ and they suggested that such isolates may have originally been enterotoxigenic but lost the plasmid during storage. During 1977 the Ohio Agriculture Research and Development Center examined 577 E. coli isolates, collected from pigs under one week old with diarrhea. They found 260 ETEC and 317 non-ETEC based

on results in pig ligated intestinal loops. Thirty-two of the enterotoxigenic strains were $K88^+$ and only one was $K99^+$ (Kohler, 1978).

It was demonstrated that production of iron-binding catechols was essential for rapid bacterial growth both in vitro and in vivo (Rogers, 1973). This type of compound was considered a virulence factor and certain strains of E. coli produced iron-binding catechols. Payne and Finkelstein (1977) used congo red agar to segregate virulent from avirulent Shigella spp., V. cholera, N. meningitidis and E. coli of human origin. Virulent colonies absorbed the congo red dye as they grew and avirulent colonies did not. This characteristic was associated with organisms ability to produce iron binding compounds.

Before the discovery of ETEC virulence factors Scherer (1966) examined E. coli cultures from bovine mastitis on Tergitol-7* medium. He recognized 6 different colony types with the aid of a dissecting microscope, (rough, intermediate rough, intermediate smooth, mucoid type A, mucoid type B and tetrazolium reducing). Rough colonies were yellow or amber, 7 to 15 mm in diameter, flat, dry and spreading, with irregular edges and had a sharp, cut-glass appearance. Intermediate rough were more compact and raised with a cut-glass appearance but were not dry or spreading. The edges were irregular and the interstices of the cut-glass portion were filled with a translucent, moist material. Intermediate smooth were compact, slightly raised, smoother and more entire with slight cut-glass surface. The colony was amber yellow in the center with lighter yellow edges. Mucoid type A colonies were

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*Difco Laboratories, Detroit, Michigan.

yellow, amber, or peach-colored mucoid globules. Mucus like strings were produced in some cultures when teased with a small wire loop. Mucoid type B colonies were small and mucus like material proliferated in a ring around the periphery when these colonies were held at room temperature. Tetrazolium reducing colonies were smaller, moist, had red centers and a clear colorless entire periphery. Blue zones were produced in the medium. He suggested that this medium might be useful for screening purposes in epidemiological studies.

MATERIALS AND METHODS

Bacterial Cultures

Seventy-nine clinical isolates, collected from different areas in Kansas from calves and pigs with diarrheal disease which were submitted to the Veterinary Diagnostic Laboratory, Kansas State University and 11 isolates from calves with diarrhea which were submitted to the Department of Laboratory Medicine were utilized. All isolates produced either ST or LT as determined by the Y-1 adrenal cell or infant mouse assays. Thirty-eight isolates were of bovine origin and 52 of porcine origin. None were serotyped.

Cultures were identified by using standard methods (Edwards and Ewing, 1972). The cultures were maintained on trypticase soy agar* (1% agar) or 2% peptone-nutrient** agar. The tubes were sealed with waxed corks and stored at room temperature in the dark until used.

E. coli strains 1474 (K12:K99^{+}), 1476 (K12:K88^{+} a.c.) and 1475 (K12) obtained from the National Animal Disease Center (NADC)***, Ames, Iowa, were utilized for antisera production. Strains 1474 and 1476 were E. coli K12 into which the plasmid for K99 or K88 was inserted.

E. coli strains 1471 (B41, 0101:K99:NM), 1472 (B44, 09:K(A) 30:K99) and 1473 (B117, 08:K85:K99) were obtained from NADC*** and strain Ø A-2 (0157:K88 a.c.:H19) from Pennsylvania State University**** were used as controls in the slide agglutination test.

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****Dr. Paul J. Glantz, Department of Veterinary Science, The Pennsylvania State University, University Park, Pennsylvania 16802

E. coli strain 263 known to produce LT and strain 1261 known to produce ST obtained from NADC* were used in enterotoxin assays as controls.

Colony Type Selection

The 90 original E. coli isolates were streaked, 6-10 at a time for single colony isolation on Tergitol-7** agar containing 1% filter sterilized 2, 3, 5-triphenyl-tetrazolium chloride** (T-7). Following incubation at 37 C for 24 hours, plates were examined under a dissecting microscope with reflected and transmitted light. A loopfull from each colony type (Scherer, 1966) was streaked on T-7 for re-examination of colony type and growth. Colonies were observed and results recorded after incubation at 37 C for 24, 48 and 72 hours and one week. A 24 hour growth of each colony type from T-7 was preserved in stock culture for additional studies and used to inoculate other media for colony morphology studies. Following selection of colony types, isolates were referred to as strains.

Colony Morphology Studies

Inoculum from a 24 hour growth of each T-7 colony type was streaked for isolation on the following media in sterile disposable plastic petri dishes.

Blood agar (BA) - Trypticase Soy Agar*** plus
5% sterile citrated bovine blood

MacConkey Agar**

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*Dr. H. W. Moon, National Animal Disease Center, Ames, Iowa.
**Difco Laboratories, Detroit, Michigan
***BioQuest, Cockeysville, Maryland

Congo Red Agar - Trypticase Soy Agar* plus
0.1 gm/liter Congo red dye
CI 22120**

Improved Minca Agar (Guinée et al., 1977)

KH_2PO_4		1.36 gm
$Na_2HPO_4 \cdot 2H_2O$		10.1 gm
Trace Salts Solution		1.0 ml
$MgSO_4 \cdot 7H_2O$	10 gm	
$MnCl_2 \cdot 4H_2O$	1 gm	
$FeCl_3 \cdot 6H_2O$	0.135 gm	
$CaCl_2 \cdot 2H_2O$	0.4 gm	
Distilled water	1000 ml	
Casamino Acids***		1.0 gm
Agar***		12.0 gm
Distilled water		1000.0 ml
IsoVitalex*		10.0 ml added

Aspetically after cooling to 45 C

Minca-Congo Red Agar - Improved Minca Agar plus 0.1 gm/liter
Congo red dye
CI21220**

Following 24, 48 and 72 hours and 1 week incubation at 37 C, colonial Characteristics on all media were examined under a dissecting microscope with reflected and transmitted light and results recorded.

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*BioQuest, Cockeysville, Maryland
**Harleco, Philadelphia, Pennsylvania
***Difco Laboratories, Detroit, Michigan

Production and Absorption of K99 and K88 Antiserums

The production of hyperimmune K88 and K99 antiserums in rabbits and methods for absorption were as described by Edwards and Ewing (1972), with the following modifications. Eschrichia coli strain 1474 (K12:K99$^+$) was used as the antigen for K99 antiserum production and E. coli strain 1476 (K12:K88ac$^+$) as the antigen for K88 antiserum production. Bacterial cells for antigen were grown on blood agar plates for 24 hours. Growth from the plates was suspended in 0.5% formalized saline for the first 2 injections and in saline for the remaining injections. The density of the suspensions for injections was as described by Edwards and Ewing (1972). Three New Zealand white rabbits were used for K99 antiserum production and 2 for K88 antiserum production. Rabbits were inoculated by the marginal ear vein with 0.3, 0.5, 0.7, 1.0, 1.3, 1.7, and 2.0 ml of the antigen suspension at 3-4 day intervals and exsanguinated 7-10 days after the last injection. Both antiserums were absorbed with the parent strain 1475 (K12) to remove heterologous antibody to all antigens of the immunizing strains except those which were coded by the K99 and K88 plasmids. Repeated absorptions of 1:10 dilutions of the antiserums were conducted until the absorbing strain would no longer react positively in a plate agglutination test with serial 2-fold dilutions of the antiserums to a dilution of 1:512. Bacterial cells for absorption were grown in a lawn on Trypticase Soy Agar*, harvested with a rubber policeman, added directly to the diluted antiserums, and evenly suspended with a vortex mixer. Growth from 20-30

*BioQuest, Cockeysville, Maryland.

plates was added to approximately 20 ml of the diluted antiserums at each absorption attempt. Three or four such absorptions were necessary to remove heterologous antibody.

Agglutination Test for K88 and K99

All colony types of each isolate were tested for the presence of K99 or K88 by agglutination on a glass plate (Ørskov et al., 1975). Each colony type was cultured on improved minca and trypticase soy agar* plates and incubated at 37 C for 24 hours. A loop full from the 24 hour cultures was suspended in 0.5 ml of formalized saline. The suspension agitated on a vortex mixer and tested for K99 and K88 antigens with monospecific antisera. The antiserum-antigen mixture was incubated at room temperature for a maximum of 8 minutes before results were read. Normal rabbit serum mixed with antigen served as a control for auto-agglutination. Negative strains were transferred daily for 4 days in tryptic soy broth** with vigorous shaking at 37 C then plated on improved minca agar and retested (Isaacson et al., 1978a).

Infant Mouse Assay for ST

Each colony type was tested for production of heat-stable enterotoxin (ST) using the infant mouse gastric test (IMGT) as described by Dean et al. (1972) and modified by Giannella (1976) with the following modifications. Groups of 3 rather than 4 mice (3-5 days old) were used. Each mouse was inoculated intragastrically with a 30 gauge needle through the abdominal wall with 0.1 ml of a crude culture containing 2 drops of

*BioQuest, Cockeysville, Maryland
**Difco Laboratories, Detroit, Michigan

0.5% Evansblue* dye per ml. Mice were incubated at room temperature (25-28 C) for 2-4 hours, and then euthanitized by chloroform inhalation. The ratio of gut weight to remaining carcass weight was calculated. A ratio of 0.083 or higher was considered positive. A ratio of 0.075 to 0.082 was considered suspicious, and the test repeated. A ratio of 0.074 or lower was considered negative.

Y-1 Adrenal Cell Assay for LT

Each T-7 colony type was tested for production of LT in Y-1 adrenal cells in miniculture as described by Sack and Sack (1975) with the following modifications. Fifty micrograms per ml of gentamycin was added to the tissue culture medium rather than 40 ug/ml. The phosphate buffered saline used to wash the cells also contained 50 ug/ml gentamycin. Whole live 24 hour cultures (0.04 ml) grown in 0.5 ml of casamino acids** -yeast extract-glucose medium (CAYE) (Mundell et al., 1976) were used to inoculate the wells of the miniculture plates***. Known positive and negative control strains were included with each plate.

Statistical Analysis

Analysis of the data was conducted by a contigency Chi-square analysis for independence.

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*Warner-Lambert Company, Morris Plains, New Jersey
**Difco Laboratories, Detroit, Michigan
***BioQuest, Cockeysville, Maryland

RESULTS

Examination of 90 isolates of ST and LT producing ETEC on Tergitol-7 (T-7) medium, resulted in recovery of 138 different strains based on colony type. Fifty-two of the original isolates produced only 1 type of colony, 29 produced 2, 8 produced 3, and 1 produced 4. The original 44 LT producing isolates segregated into 70 strains and the 46 ST producing isolates into 68 (Table 1). The 9 bovine LT producing isolates segregated into 14 strains (1:1.6) (Table 2), 35 porcine LT isolates into 56 strains (1:1.6) (Table 3), 29 bovine ST isolates into 40 strains (1:1.4) (Table 2) and 17 porcine ST isolates into 28 strains (1:1.6) (Table 3). All colony types described by Scherer (1966) were recognized (Table 1). Most (83%) of the strains produced colonies that were classified as either mucoid-A, mucoid-B, or intermediate smooth (Table 1). When the strains were restreaked onto T-7 they retained colony morphology.

Colonies on blood agar were very similar, regardless of the colony types on T-7. Rough and intermediate type colonies tended to have a grayish-white translucent periphery with a dense white center and mucoid-A were whiter and denser than intermediate or rough types. Tetrazolium reducing colonies were smaller in diameter. Colonial morphology on blood agar did not predict colony type on T-7. Forty-eight porcine and nine bovine strains produced complete hemolysis. All but seven of these produced 1 or more virulence factors. Thirty-one of these strains produced mucoid-B colonies and 15 intermediate smooth on T-7. Of the 64 porcine strains which produced 1 or more virulence factors, 43 (67.2%) were hemolytic (Table 4).

TABLE 1. Segregation of 90 Bovine and Porcine Enterotoxin Producing E. coli Original Isolates into Colony Type Strains on Tergitol-7 Agar.

Colony Types	Number of Colony Types (CT)					
	Heat Labile Toxin Producing			Heat Stable Toxin Producing		
	24 Isolates 1 CT[a] Each	15 Isolates 2 CT Each	5 Isolates 3 or 4 CT Each	28 Isolates 1 CT Each	14 Isolates 2 CT Each	4 Isolates 3 CT Each
Rough	1	3	3	0	6	3
Mucoid-A	2	2	0	21	13	5
Mucoid-B	16	12	6	1	0	0
Intermediate Smooth	4	13	4	4	7	2
Intermediate Rough	1	0	3	2	1	1
Tetrazolium Reducing	0	0	0	0	1	1
Total	24	30	16	28	28	12

a - Colony Type

TABLE 2. Segregation of 38 Bovine Enterotoxin Producing E. coli Original Isolates into Colony Type Strains on Tergitol-7 Agar.

Colony Types	Number of Colony Types (CT)					
	Heat Labile Toxin Producing			Heat Stable Toxin Producing		
	6 Isolates 1 CT[a] Each	2 Isolates 2 CT Each	1 Isolates 4 CT Each	20 Isolates 1 CT Each	7 Isolates 2 CT Each	2 Isolates 3 CT Each
Rough	0	2	1	0	2	1
Mucoid-A	1	0	0	16	7	3
Mucoid-B	2	0	1	0	0	0
Intermediate Smooth	3	2	1	2	4	1
Intermediate Rough	0	0	1	2	1	1
Tetrazolium Reducing	0	0	0	0	0	0
Total	6	4	4	20	14	6

a - Colony Type

TABLE 3. Segregation of 52 Porcine Enterotoxin Producing E. coli Original Isolates into Colony Type Strains on Tergitol-7 Agar.

Colony Types	Number of Colony Types (CT)					
	Heat Labile Toxin Producing			Heat Stable Toxin Producing		
	18 Isolates 1 CT[a] Each	13 Isolates 2 CT Each	4 Isolates 3 CT Each	8 Isolates 1 CT Each	7 Isolates 2 CT Each	2 Isolates 3 CT Each
Rough	1	1	2	0	4	2
Mucoid-A	1	2	0	5	6	2
Mucoid-B	14	12	5	1	0	0
Intermediate Smooth	1	11	3	2	3	1
Intermediate Rough	1	0	2	0	0	0
Tetrazolium Reducing	0	0	0	0	1	1
Total	18	26	12	8	14	6

a - Colony Type

TABLE 4. Correlation of Virulence Factor Production by Hemolytic Porcine E. coli with Colony Type on Tergitol-7 Agar.

Colony Type	Virulence Factors Produced											
	ST[a]/K99[b]	LT[c]/K88[d]	ST	LT	K99	K88	ST/LT/ K99	ST/LT/ K88	ST/LT	Sub Total	None	Total
Rough	0	0	0	1	0	0	0	0	0	1	1	2
Mucoid-A	0	0	0	0	0	0	0	0	0	0	0	0
Mucoid-B	0	24	0	1	0	2	0	1	1	29	1	30
Intermediate Smooth	1	0	0	11	0	0	1	0	0	13	2	15
Intermediate Rough	0	0	0	0	0	0	0	0	0	0	1	1
Tetrazolium Reducing	0	0	0	0	0	0	0	0	0	0	0	0
Total	1	24	0	13	0	2	1	1	1	43	5	48

a - Heat stable enterotoxin
b - Colonization antigen K99
c - Heat labile enterotoxin
d - Colonization antigen K88

Tetrazolium reducing colonies were colorless on MacConkey agar. All others produced typical red-pink, lactose fermenting colonies. Rough and intermediate type colonies were low convex, circular, with an entire edge and a slightly rough surface. Mucoid-A colonies were more viscous than others and tended to string out when teased with a loop.

Minca agar colonies, regardless of the T-7 colony type were similar. They were low convex, smooth, with an entire edge and glistening. The center of the colony was milky white and the periphery translucent. Mucoid-A colonies also produced mucus-like strings when teased with a loop on the medium.

Colony types on congo red agar were similar in appearance. All except rough were smooth, convex, with an entire edge, pink center and a creamy pink periphery. Many of these colonies were mucoid. Rough colonies were flat, dry, granular, and had an irregular edge. Five strains absorbed the dye within the first 24 hours of growth. The remainder required 2-3 days incubation to become red.

All minca congo red colonies except those which were rough were similar in appearance. They were slightly larger and less convex than on congo red. Rough colonies were flat, dry, granular and had an irregular periphery. The congo red dye was absorbed into the colony more rapidly than on congo red agar, but no difference between strains in length of time required for absorption was noted.

Thirty-two of the bovine and 15 of the porcine strains produced ST (Tables 5 and 6). These strains produced all T-7 colony types. However, 28 of the bovine and 12 porcine ST^+ strains produced either mucoid-A or

TABLE 5. Correlation of Virulence Factor Production by Porcine E. coli with Colony Type on Tergitol-7 Agar.

Colony Type	Virulence Factors Produced											
	ST[a]/K99[b]	LT[c]/K88[d]	ST	LT	K99	K88	ST/LT/ K99	ST/LT/ K88	ST/LT	Sub Total	None	Total
Rough	1	0	1	1	0	0	0	0	0	3	7	10
Mucoid-A	3	0	7	0	0	0	0	0	0	10	6	16
Mucoid-B	0	25	0	1	0	2	0	1	1	30	1	31
Intermediate Smooth	2	0	0	14	1	1	1	0	0	19	3	22
Intermediate Rough	0	0	0	1	0	0	0	0	0	1	2	3
Tetrazolium Reducing	0	0	1	0	0	0	0	0	0	1	1	1
Total	6	25	9	17	1	3	1	1	1	64	20	84

a - Heat stable enterotoxin
b - Colonization antigen K99
c - Heat labile enterotoxin
d - Colonization antigen K88

TABLE 6. Correlation of Virulence Factor Production by Bovine E. coli with Colony Type on Tergitol-7 Agar.

Colony Type	Virulence Factors Produced					
	ST[a]/K99[b]	ST	LT[c]	Sub Total	None	Total
Rough	3	0	1	4	2	6
Mucoid-A	22	1	0	23	4	27
Mucoid-B	0	0	3	3	0	3
Intermediate Smooth	5	0	3	8	5	13
Intermediate Rough	0	1	1	2	3	5
Total	30	2	8	40	14	54

a - Heat stable enterotoxin
b - Colonization antigen K99
c - Heat labile enterotoxin

intermediate smooth colonies on T-7. These represented 85.1% of all ST producing strains. Five bovine and 4 porcine original isolates which segregated into 6 bovine and 4 porcine strains produced ST when first examined, but failed to react on re-examination (Appendix Tables 1 and 3). Twenty-two original ST^+ isolates (16 bovine and 6 porcine) produced only one type of colony on T-7 and were all ST^+ on re-examination. Nine original ST^+ isolates (7 bovine and 2 porcine) produced two or three types of colony on T-7 and all colony types were ST^+ on re-examination. Six original ST^+ isolates (1 bovine and 5 porcine) produced two or three types of colony on T-7 but were ST^+ on re-examination on only 1 or 2 of the colony types. Correlation of ST production with colony type on T-7 without regard to species is presented in Table 7.

Eight of the bovine and 42 of the porcine strains produced LT only (Table 5 and 6). These strains produced rough, mucoid-B, intermediate smooth and intermediate rough colonies. However, only 2 bovine and 2 porcine strains produced colonies which were not mucoid-B or intermediate smooth. These two types of colonies represented 92% of all LT producing strains. Four bovine and 5 porcine original isolates which segregated into 6 bovine and 6 porcine strains produced LT when first examined, but failed to react on re-examination (Appendix Tables 2 and 4). Eighteen original LT positive (LT^+) isolates (4 bovine and 14 porcine) produced only one type of colony on T-7 and were all LT^+ on re-examination. Thirteen original LT^+ isolates (12 porcine and 1 bovine) produced two, three, or four types of colony on T-7 and all colony types were LT^+ on re-examination. Four original LT^+ porcine isolates produced two or three types of colony on T-7 but were LT^+ on re-examination on only one

TABLE 7. Correlation of Virulence Factor Production by Bovine and Porcine E. coli with Colony Type on Tergitol-7 Agar.

Colony Type	Virulence Factors Produced											
	ST[a]/K99[b]	LT[c]/K88[d]	ST	LT	K99	K88	ST/LT/ K99	ST/LT/ K88	ST/LT	Sub Total	None	Total
Rough	4	0	1	2	0	0	0	0	0	7	9	16
Mucoid-A	25	0	8	0	0	0	0	0	0	33	10	43
Mucoid-B	0	25	0	4	0	2	0	1	1	33	1	34
Intermediate Smooth	7	0	0	17	1	1	1	0	0	27	8	35
Intermediate Rough	0	0	1	2	0	0	0	0	0	3	5	8
Tetrazolium Reducing	0	0	1	0	0	0	0	0	0	1	1	2
Total	36	25	11	25	1	3	1	1	1	104	34	138

a - Heat stable enterotoxin
b - Colonization antigen K99
c - Heat labile enterotoxin
d - Colonization antigen K88

of the colony types. Correlation of LT production with colony type on T-7 without regard to species is presented in Table 7.

Three porcine strains produced both ST and LT (Table 5). Two of these strains were mucoid-B and one was intermediate smooth on T-7. The intermediate smooth strain was originally an ST producer and formed only one colony type on T-7. One of the mucoid-B strains was originally an LT producer and it formed only one colony type on T-7. The other mucoid-B strain was originally an LT producer, but on T-7 it formed both mucoid-B and intermediate smooth colonies. The intermediate smooth strain produced only LT but the mucoid-B strain produced both ST and LT.

Thirty-eight strains failed to produce either LT or ST. Twenty-five of these were strains of those 18 original ST or LT positive isolates which failed to react on re-examination. The remaining 13 represented strains of 10 original isolates which produced more than one type of colony on T-7 and one of the other colony types was either ST or LT positive.

Thirty bovine and 8 porcine strains possessed the K99 antigen (Tables 5 and 6). These strains produced only rough, mucoid-A and intermediate smooth colonies. However, only 3 bovine and 1 porcine strains produced colonies which were not either mucoid-A or intermediate smooth. These two types of colonies represented 84.2% of all $K99^+$ strains. Eighteen of the original isolates produced only one type of colony on T-7 and were $K99^+$. Seven of the original isolates (6 bovine and 1 porcine) produced two or three types of colonies on T-7 and were $K99^+$ on all colony types. Four of the original isolates (1 bovine and 3 porcine) produced two or three types of colonies on T-7 and were $K99^+$

on only 1 of the colony types. Correlation of $K99^+$ strains with colony type on T-7 without regard to species is presented in Table 7.

Twenty-nine porcine strains possessed the K88 antigen. One of these strains was intermediate smooth and the rest mucoid-B on T-7 (Table 5). The strain which was $K88^+$ and formed an intermediate smooth colony on T-7 was different from all other $K88^+$ strains. The original isolate (5483) from which it was derived produced ST and two T-7 colony types. This strain did not produce ST, but the other strain of the original isolate did produce ST (Appendix Table 3). Thirteen of the original isolates produced only one type of colony on T-7 and were $K88^+$. Sixteen of the original isolates produced two or three types of colonies on T-7 and were $K88^+$ on only 1 of the colony types (Appendix Tables 3 and 4). Correlation of $K88^+$ strains with colony types on T-7 without regard to species is presented in Table 7.

Seventy-one strains failed to produce either the K88 or K99 colonizing antigen (Table 7). Thirty-four of these were accounted for in the strains which did not produce any of the virulence factors. Thirty-seven of these strains produced ST, LT or both but not either of the colonizing antigens (Table 7). Sixty-three of the 100 strains which were also enterotoxin producers possessed either K88 or K99.

Thirty bovine strains and 6 porcine strains possessed the K99 antigen in combination with the ability to produce ST (Tables 5 and 6). These strains produced rough, mucoid-A and intermediate smooth colonies on T-7. However, only 3 bovine and 1 porcine strains produced rough colonies. The intermediate smooth and mucoid-A colony producing strains represented 88.9% of the strains with this combination of virulence

factors (Table 7).

Twenty-five porcine strains possessed the K88 antigen in combination with the ability to produce LT (Table 5). These strains produced only mucoid-B colonies on T-7.

Two of the 3 porcine cultures which produced both ST and LT also possessed colonizing antigens. One had K88 and the other K99 (Table 5).

Analysis of the groups which formed based on colony morphology on T-7 indicated that mucoid-A colonies of bovine origin with ST/K99, mucoid-B of porcine origin with LT/K88 and intermediate smooth of porcine origin with LT were statistically different ($P \ll 6.001$) from all other classes. Twenty-two of 27 bovine strains which produced mucoid-A colonies were also ST/K99 positive. Ninety-five percent confidence intervals for the combination of these characteristics were 66.8 to 96.1%. Twenty-five of 31 porcine strains which produced mucoid-B colonies were also LT/K88 positive. Ninety-five percent confidence intervals for the combination of these characteristics were 66.7 to 94.6%. Fourteen of 22 porcine strains which produced intermediate smooth colonies were also LT positive. Ninety-five percent confidence intervals for the combination of these characteristics were 43.5 to 83.7%. Ninety-three of 112 (83%) strains of bovine and porcine origin which produced either mucoid-A, mucoid-B, or intermediate smooth colonies also possessed one or more of the virulence factors. Ninety-five percent confidence intervals for combination of these colony types with a virulence factor were 76.1 to 90%.

Trypticase soy agar* and minca were equally effective as a growth

*BioQuest, Cockeysville, Md.

medium for serologic detection of K88. However, when grown on TSA cells tended to autoagglutinate. Eight K99 and one K88 positive strains which were not detected on minca agar were detected after transfer for 4 days in shaken trypticase soy broth followed by culturing on minca agar.

DISCUSSION

Previous reports concerning the relationship between E. coli colony morphology and their virulence factors were inconsistent and concerned primarily with detection of surface antigens (Ørskov et al., 1975 and Moon et al., 1976). Surface antigens and enterotoxin production were the two major attributes of virulence that contributed to enteropathogenicity of E. coli of calves and pigs (Isaacson et al., 1978a; Moon et al., 1976; Jones and Rutter, 1972; Guinée et al., 1977; and Burrows et al., 1976). Presence of one or the other of the virulence factors indicated, but not conclusively, enteropathogenicity of strains, since some non-enterotoxigenic strains produced only the surface antigens (Moon et al., 1976; and Isaacson et al., 1978a). Both factors were required for production of severe diarrhea (Smith and Linggood, 1971). This study correlated not only surface antigens but also enterotoxin production with colony morphology.

Tergitol-7 agar was the most useful of the media examined for differentiation of E. coli colony types. All six of the colony types described by Scherer (1966) were recognized and there was at least a degree of association between colony type and production of virulence factors on T-7 agar. Ninety-three of the 104 (89.4%) strains which produced one or more of the virulence factors produced either mucoid-A, mucoid-B, or intermediate smooth colonies on T-7. Nineteen strains which did not produce virulence factors also produced these types of colonies. Of these, 5 were strains of original isolates which produced more than one type of colony on T-7. At least one of the other strains

produced mucoid-A, mucoid-B or intermediate smooth colonies and produced one or more of the virulence factors (Appendix Tables 1, 2, 3 and 4).

The results indicated that there was a high degree of association between mucoid-A colonies and ST production or a combination of ST production and possession of K99. Thirty-three of 43 (76.7% colonies of this type produced ST and 25 of 43 (58.1%) were both ST and K99 positive (Table 7). Strains of bovine origin correlated better than did those of porcine origin (Tables 5 and 6). This result would be anticipated, since calf ETEC are mostly ST/K99 positive and porcine ETEC LT/K88 positive (Moon et al., 1976; Isaacson et al., 1978a; Smith and Linggood, 1971b).

Association of mucoid-B type colonies with production of LT and/or K88 was pronounced (Table 7). Thirty-three of 34 (97.1%) of colonies of this type produced LT and/or K88. One of these was positive for ST, LT and K88 and another for ST and LT. Most strains in this category were of porcine origin (Table 5). Only 3 bovine strains produced LT and none were K88 positive (Table 6). This result would also be anticipated since bovine strains are more frequently ST/K99 positive (Isaacson et al., 1978a, and Moon et al., 1976).

An additional high degree of association was between intermediate smooth colonies and LT production. Eighteen of 35 (51.4%) strains of this colony type produced LT (Table 7). One of these strains was ST, LT and K99 positive. Strains in this class were predominantly of porcine origin (Tables 5 and 6).

A possible relationship between the mucoid-B/LT/K88 class and the intermediate smooth/LT class was noted. Most strains which were both

LT and K88 positive produced mucoid-B colonies and most strains which were only LT positive were intermediate smooth (Table 7). Strains may spontaneously loose the plasmid which codes either of these traits (Gyles et al., 1974b; Smith and Linggood, 1971a and Ørskov and Ørskov, 1966), and to be highly enteropathogenic strains must carry both (Smith and Linggood, 1971b). When the plasmid which codes for K88 production is lost, the colony type on T-7 may switch from mucoid-B to intermediate smooth.

A relationship of diagnostic importance between hemolysis and enteropathogenicity of porcine, but not bovine E. coli has been previously noted (Sojka, 1965). Somatic (O) serogroups 138, 139,141, and 8 tended to be hemolytic more often than did other serogroups.

In this study, hemolysis on blood agar correlated with virulence factor production in 43 of 64 (67.2%) porcine strains (Table 4). This characteristic was observed more frequently with strains which produced mucoid-B or intermediate smooth colonies on T-7. Few bovine strains were hemolytic and those that were did not appear to correlate with any virulence factor or colony type on T-7.

Colony characteristics on other media did not appear useful for selection of virulence factors. Congo red agar was used by Payne and Finkelstein (1977) to differentiate virulent and avirulent strains of E.coli. However, the strains they utilized were of human origin and may have been Shigella-like E. coli which are locally enteroinvasive (Formal et al., 1978). This type of pathogenic mechanism has not been demonstrated to occur in enteropathogenic E. coli strains from domestic animals (Gyles, 1978). It is possible that the same unrecognized

factors which exclude Shigella sp. as pathogens in domestic animals also exclude this type of E. coli as pathogens.

This study confirmed the results of Guinée et al. (1977) where improved minca medium improved the detectability of the K99 antigen. None of the 138 strains agglutinated in monospecific K99 antiserum when grown on trypticase soy agar*. Thirty-eight did when grown on improved minca. The method described by Isaacson et al. (1978a) of transferring cultures daily for 4 days in trypticase soy broth with vigorous shaking followed by plating on improved minca also improved the detectability of the K99 antigen. Eight K99 and one K88 positive strains were detected only by this method.

This study failed to demonstrate either ST or LT production on 18 of the isolates although they originally produced enterotoxin. Possible reasons for these failures are multiple. Extrachromosomal DNA is not stable and the plasmids which code for these characteristics may be lost spontaneously during storage or manipulation of cultures (Ørskov and Ørskov, 1966, and Jones and Rutter, 1972). False positives may have occurred when the isolates were first tested or false negatives occurred when retested. An additional possibility is that when cultures were subcultured, enterotoxin negative varients were selected.

The purpose of this study was to determine if any of the known E. coli virulence factors would be reflected in colony morphology. Therefore, only cultures which were known to possess at least one of these characteristics were selected as subjects. No attempt was made

*BioQuest, Cockeysville, Md.

to examine non-enteropathogenic strains from calves or pigs nor was any intentional attempt made to compare the strains in this study which produced virulence factors to those which did not. This study indicated that many of those isolates which possessed one or more of the virulence factors were hemolytic on blood agar and produced either mucoid-A, mucoid-B or intermediate smooth colonies on Tergitol-7 agar. Therefore, it is suggested that these two types of media may be helpful in preliminary examination for enteropathogenic E. coli from calves or pigs.

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APPENDIX

Tables 1, 2, 3 and 4 Legend

a - Ent., Enterotoxin

b - ST, Heat Stable Enterotoxin

c - LT, Heat Labile Enterotoxin

d - K99, Colonizing Antigen K99

e - K88, Colonizing Antigen K88

f - B.W., Body Weight

g - G.W., Gut Weight

h - Minca, Improved Minca Medium

i - TSA, Trypticase Soy Agar*

j - TSB, Tryptic Soy Broth*

*BioQuest, Cockeysville, Maryland

TABLE 1. Colony Morphology on Tergitol-7 Agar and Virulence Factor Production by E. coli Heat Stable Enterotoxin Producing Cultures of Bovine Origin.

Strain Number	Original Isolate Ent.[a]	Colonies On Tergitol-7		Infant Mouse Assay-ST[b]				Y-1 Adrenal Cell-LT[c]	Ent. Produced	Plate Agglutination Test					
										For K99[d]			For K88[e]		
		Number	Type	B.W.[f] gm.	G.W.[g] gm.	Ratio	Result			Minca[h]	TSA[i]	TSB[j] Minca	Minca	TSA	TSB Minca
5258	ST	A	MA	12.650	0.787	0.062	-	-	-	-	-	-	-	-	-
5356	ST	A	IR	7.356	0.446	0.061	-	-	-	-	-	-	-	-	-
3609	ST	A	IR	12.720	0.760	0.060	-	-	-	-	-	-	-	-	-
6179	ST	A	MA	9.240	0.760	0.082	-	-	-	-	-	-	-	-	-
4116	ST	A	MA	7.981	0.918	0.115	+	-	ST	+	-	NT	-	-	-
5266	ST	A	IS	7.019	0.839	0.120	+	-	ST	+	-	NT	-	-	-
-138	ST	A	MA	8.840	1.090	0.123	+	-	ST	+	-	NT	-	-	-
-133	ST	A	MA	9.190	1.520	0.165	+	-	ST	+	-	NT	-	-	-
-143	ST	A	MA	8.350	0.980	0.117	+	-	ST	+	-	NT	-	-	-
-141	ST	A	MA	10.760	1.390	0.129	+	-	ST	+	-	NT	-	-	-
-139	ST	A	MA	8.680	1.300	0.149	+	-	ST	+	-	NT	-	-	-
-137	ST	A	MA	8.240	1.050	0.127	+	-	ST	+	-	NT	-	-	-
-140	ST	A	MA	4.010	0.540	0.134	+	-	ST	+	-	NT	-	-	-

Table 1 (continued)

Strain Number	Original Isolate Ent.[a]	Colonies On Tergitol-7		Infant Mouse Assay-ST[b]				Y-1 Adrenal Cell-LT[c]	Ent. Produced	Plate Agglutination Test					
										For K99[d]			For K88[e]		
		Number	Type	B.W.[f] gm.	G.W.[g] gm.	Ratio	Result			Minca[h]	TSA[i]	TSB Minca[j]	Minca	TSA	TSB Minca
-135	ST	A	MA	8.020	0.830	0.103	+	-	ST	+	-	NT	-	-	-
6245	ST	A	MA	9.155	1.212	0.132	+	-	ST	-	-	+	-	-	-
6237	ST	A	MA	10.136	1.193	0.118	+	-	ST	+	-	NT	-	-	-
6303	ST	A	IS	11.091	1.495	0.135	+	-	ST	+	-	NT	-	-	-
6132	ST	A	MA	9.077	1.240	0.136	+	-	ST	-	-	+	-	-	-
6081	ST	A	MA	9.971	1.330	0.133	+	-	ST	-	-	+	-	-	-
5982	ST	A	MA	9.422	1.250	0.132	+	-	ST	+	-	NT	-	-	-
-136	ST	A	MA	10.380	0.690	0.066	-	-	-	-	-	-	-	-	-
		B	IS	11.170	0.780	0.070	-	-	-	-	-	-	-	-	-
5368	ST	A	MA	5.833	0.853	0.146	+	-	ST	-	-	-	-	-	-
		B	IR	7.927	1.006	0.127	+	-	ST	-	-	-	-	-	-
5200	ST	A	MA	7.650	0.740	0.097	+	-	ST	+	-	NT	-	-	-
		B	R	7.060	0.753	0.107	+	-	ST	+	-	NT	-	-	-

Table 1 (continued)

Strain Number	Original Isolate Ent.[a]	Colonies On Tergitol-7		Infant Mouse Assay-ST[b]				Y-1 Adrenal Cell-LT[c]	Ent. Produced	Plate Agglutination Test					
										For K99[d]			For K88[e]		
		Number	Type	B.W.[f] gm.	G.W.[g] gm.	Ratio	Result			Minca[h]	TSA[i]	TSB[j] Minca	Minca	TSA	TSB Minca
3558	ST	A	MA	3.895	0.470	0.121	+	-	ST	+	-	NT	-	-	-
		B	R	9.233	1.255	0.136	+	-	ST	+	-	NT	-	-	-
-142	ST	A	MA	8.850	1.180	0.133	+	-	ST	-	-	+	-	-	-
		B	IS	8.970	1.140	0.127	+	-	ST	+	-	NT	-	-	-
-134	ST	A	IS	13.800	1.800	0.130	+	-	ST	+	-	NT	-	-	-
		B	MA	11.190	1.460	0.130	+	-	ST	+	-	NT	-	-	-
6214	ST	A	MA	9.758	1.261	0.129	+	-	ST	-	-	-	-	-	-
		B	IS	10.118	1.273	0.126	+	-	ST	+	-	NT	-	-	-
2714	ST	A	R	14.236	1.480	0.104	+	-	ST	+	-	NT	-	-	-
		B	MA (Peach)	7.303	0.814	0.111	+	-	ST	+	-	NT	-	-	-
		C	MA (Yellow)	8.260	1.200	0.145	+	-	ST	+	-	NT	-	-	-
2779	ST	A	IS	8.630	0.510	0.059	-	-	-	-	-	-	-	-	-
		B	MA	9.150	0.800	0.087	+	-	ST	-	-	+	-	-	-
		C	IR	9.130	0.610	0.067	-	-	-	-	-	-	-	-	-

TABLE 2. Colony Morphology on Tergitol-7 Agar and Virulence Factor Production by E. coli Heat Labile Enterotoxin Producing Cultures of Bovine Origin.

Strain Number	Original Isolate Ent.[a]	Colonies On Tergitol-7		Infant Mouse Assay-ST[b]				Y-1 Adrenal Cell-LT[c]	Ent. Produced	Plate Agglutination Test					
										For K99[d]			For K88[e]		
		Number	Type	B.W.[f] gm.	G.W.[g] gm.	Ratio	Result			Minca[h]	TSA[i]	TSB[j] Minca	Minca	TSA	TSB Minca
5090	LT	A	MA	6.459	0.416	0.063	-	-	-	-	-	-	-	-	-
3697	LT	A	IS	8.434	0.490	0.058	-	-	-	-	-	-	-	-	-
3855	LT	A	MB	9.928	0.572	0.058	-	+	LT	-	-	-	-	-	-
4102	LT	A	IS	6.453	0.466	0.072	-	+	LT	-	-	-	-	-	-
3388	LT	A	IS	5.340	0.320	0.060	-	+	LT	-	-	-	-	-	-
5489	LT	A	MB	5.095	0.238	0.047	-	+	LT	-	-	-	-	-	-
3987	LT	A	IS	7.356	0.465	0.063	-	-	-	-	-	-	-	-	-
		B	R	7.948	0.495	0.063	-	-	-	-	-	-	-	-	-
3759	LT	A	IS	9.440	0.583	0.062	-	-	-	-	-	-	-	-	-
		B	R	8.865	0.605	0.068	-	-	-	-	-	-	-	-	-
3566	LT	A	MB	9.456	0.558	0.059	-	+	LT	-	-	-	-	-	-
		B	IS	14.370	0.881	0.062	-	+	LT	-	-	-	-	-	-

Table 2 (continued)

Strain Number	Original Isolate Ent.[a]	Colonies On Tergitol-7		Infant Mouse Assay-ST[b]				Y-1 Adrenal Cell-LT[c]	Ent. Produced	Plate Agglutination Test					
										For K99[d]			For K88[e]		
		Number	Type	B.W.[f] gm.	G.W.[g] gm.	Ratio	Result			Minca[h]	TSA[i]	TSB Minca[j]	Minca	TSA	TSB Minca
		C	R	7.768	0.495	0.064	-	+	LT	-	-	-	-	-	-
		D	IR	13.134	0.737	0.056	-	+	LT	-	-	-	-	-	-

TABLE 3. Colony Morphology on Tergitol-7 Agar and Virulence Factor Production by *E. coli* Heat Stable Enterotoxin Producing Cultures of Porcine Origin.

Strain Number	Original Isolate Ent.[a]	Colonies On Tergitol-7		Infant Mouse Assay-ST[b]				Y-1 Adrenal Cell-LT[c]	Ent. Produced	Plate Agglutination Test					
										For K99[d]			For K88[e]		
		Number	Type	B.W.[f] gm.	G.W.[g] gm.	Ratio	Result			Minca[h]	TSA[i]	TSB[j] Minca	Minca	TSA	TSB Minca
4954	ST	A	MB	7.213	0.522	0.072	-	-	-	-	-	-	-	-	-
5983	ST	A	MA	10.572	0.700	0.060	-	-	-	-	-	-	-	-	-
4994	ST	A	MA	6.627	0.787	0.118	+	-	ST	-	-	-	-	-	-
5438	ST	A	MA	6.517	0.774	0.119	+	-	ST	-	-	-	-	-	-
5556	ST	A	MA	8.260	0.880	0.107	+	-	ST	-	-	-	-	-	-
5788	ST	A	MA	8.160	1.090	0.134	+	-	ST	-	-	-	-	-	-
5491	ST	A	IS	6.356	0.700	0.110	+	-	ST	+	-	NT	-	-	-
5645	ST	A	IS	6.930	1.015	0.146	+	+	ST/LT	-	-	+	-	-	-
5532	ST	A	MA	8.690	0.540	0.062	-	-	-	-	-	-	-	-	-
		B	R	7.821	0.540	0.069	-	-	-	-	-	-	-	-	-
5419	ST	A	R	7.820	0.490	0.063	-	-	-	-	-	-	-	-	-
		B	IS	7.303	0.949	0.130	+	-	ST	+	-	NT	-	-	-
5483	ST	A	IS	9.120	0.620	0.068	-	-	-	-	-	-	+	+	+
		B	RE-S	3.461	0.292	0.085	+	-	ST	-	-	-	-	-	-

Table 3 (continued)

Strain Number	Original Isolate Ent.[a]	Colonies On Tergitol-7		Infant Mouse Assay-ST[b]				Y-1 Adrenal Cell-LT[c]	Ent. Produced	Plate Agglutination Test					
										For K99[d]			For K88[e]		
		Number	Type	B.W.[f] gm.	G.W.[g] gm.	Ratio	Result			Minca[h]	TSA[i]	TSB[j] Minca	Minca	TSA	TSB Minca
5984	ST	A	R	8.743	0.650	0.074	-	-	-	-	-	-	-	-	-
		B	MA	7.965	1.040	0.130	+	-	ST	+	-	NT	-	-	-
6164	ST	A	MA	8.282	1.020	0.123	+	-	ST	+	-	NT	-	-	-
		B	IS	10.288	0.650	0.063	-	-	-	-	-	+	-	-	-
5312	ST	A	MA	3.460	0.400	0.116	+	-	ST	-	-	-	-	-	-
		B	R	5.610	0.570	0.102	+	-	ST	-	-	-	-	-	-
5700	ST	A	MA (Yellow)	8.693	1.002	0.115	+	-	ST	-	-	-	-	-	-
		B	MA (Peach)	6.177	0.864	0.140	+	-	ST	-	-	-	-	-	-
4922	ST	A	R	10.446	0.625	0.060	-	-	-	-	-	-	-	-	-
		B	IS	9.490	0.560	0.059	-	-	-	-	-	-	-	-	-
		C	RE-S	10.560	0.603	0.057	-	-	-	-	-	-	-	-	-
4899	ST	A	MA (Peach)	11.675	0.698	0.060	-	-	-	-	-	-	-	-	-
		B	MA (Yellow)	10.805	1.146	0.106	+	-	ST	+	-	NT	-	-	-
		C	R	7.363	0.868	0.117	+	-	ST	+	-	NT	-	-	-

TABLE 4. Colony Morphology on Tergitol-7 Agar and Virulence Factor Production by E. coli Heat Labile Enterotoxin Producing Cultures of Porcine Origin.

Strain Number	Original Isolate Ent.[a]	Colonies On Tergitol-7		Infant Mouse Assay-ST[b]				Y-1 Adrenal Cell-LT[c]	Ent. Produced	Plate Agglutination Test					
										For K99[d]			For K88[e]		
		Number	Type	B.W.[f] gm.	G.W.[g] gm.	Ratio	Result			Minca[h]	TSA[i]	TSB[j] Minca	Minca	TSA	TSB Minca
5651	LT	A	R	10.135	0.673	0.066	-	-	-	-	-	-	-	-	-
5874	LT	A	MA	10.070	0.750	0.074	-	-	-	-	-	-	-	-	-
5835	LT	A	MB	10.060	0.700	0.069	-	-	-	-	-	-	+	+	NT
6257	LT	A	MB	11.905	0.714	0.059	-	-	-	-	-	-	+	+	NT
5241	LT	A	IR	7.350	0.449	0.061	-	+	LT	-	-	-	-	-	-
5801	LT	A	IS	8.430	0.570	0.068	-	+	LT	-	-	-	-	-	-
5994	LT	A	MB	9.670	0.850	0.088	+	+	ST/LT	-	-	-	-	-	-
4996	LT	A	MB	8.494	0.492	0.058	-	+	LT	-	-	-	+	+	NT
5496	LT	A	MB	7.587	0.354	0.047	-	+	LT	-	-	-	+	+	NT
5767	LT	A	MB	8.463	0.504	0.060	-	+	LT	-	-	-	+	+	NT
5672	LT	A	MB	7.421	0.478	0.064	-	+	LT	-	-	-	+	+	NT
5866	LT	A	MB	10.060	0.720	0.072	-	+	LT	-	-	-	+	+	NT
6020	LT	A	MB	7.340	0.450	0.061	-	+	LT	-	-	-	+	+	NT
6188	LT	A	MB	7.860	0.540	0.069	-	+	LT	-	-	-	+	+	NT

Table 4 (continued)

Strain Number	Original Isolate Ent.[a]	Colonies On Tergitol-7		Infant Mouse Assay-ST[b]				Y-1 Adrenal Cell-LT[c]	Ent. Produced	Plate Agglutination Test					
										For K99[d]			For K88[e]		
		Number	Type	B.W.[f] gm.	G.W.[g] gm.	Ratio	Result			Minca[h]	TSA[i]	TSB Minca[j]	Minca	TSA	TSB Minca
6170	LT	A	MB	9.502	0.660	0.069	-	+	LT	-	-	-	+	+	NT
6052	LT	A	MB	9.164	0.660	0.072	-	+	LT	-	-	-	+	+	NT
6091	LT	A	MB	8.528	0.610	0.071	-	+	LT	-	-	-	+	+	NT
6100	LT	A	MB	9.043	0.560	0.060	-	+	LT	-	-	-	+	+	NT
6390	LT	A	R	15.844	0.987	0.062	-	-	-	-	-	-	-	-	-
		B	MA	6.779	0.461	0.068	-	-	-	-	-	-	-	-	-
5031	LT	A	MA	4.519	0.267	0.059	-	-	-	-	-	-	-	-	-
		B	MB	6.224	0.405	0.065	-	+	LT	-	-	-	+	+	NT
5972	LT	A	IS	8.110	0.560	0.069	-	-	-	-	-	-	-	-	-
		B	MB	11.070	0.820	0.074	-	+	LT	-	-	-	+	+	NT
4946	LT	A	MB	9.181	0.305	0.033	-	+	LT	-	-	-	+	+	NT
		B	IS	7.986	0.506	0.063	-	+	LT	-	-	-	-	-	-
5337	LT	A	IS	5.690	0.320	0.056	-	+	LT	-	-	-	-	-	-
		B	MB	7.002	0.428	0.061	-	+	LT	-	-	-	+	+	NT

Table 4 (continued)

Strain Number	Original Isolate Ent.[a]	Colonies On Tergitol-7		Infant Mouse Assay-ST[b]				Y-1 Adrenal Cell-LT[c]	Ent. Produced	Plate Agglutination Test					
										For K99[d]			For K88[e]		
		Number	Type	B.W.[f] gm.	G.W.[g] gm.	Ratio	Result			Minca[h]	TSA[i]	TSB[j] Minca	Minca	TSA	TSB Minca
5544	LT	A	MB	9.800	0.640	0.065	-	+	LT	-	-	-	+	+	NT
		B	IS	7.965	0.429	0.054	-	+	LT	-	-	-	-	-	-
5301	LT	A	MB	6.275	0.324	0.052	-	+	LT	-	-	-	+	+	NT
		B	IS	11.564	0.710	0.061	-	+	LT	-	-	-	-	-	-
6173	LT	A	IS	7.540	0.430	0.057	-	+	LT	-	-	-	-	-	-
		B	MB	7.490	0.500	0.067	-	+	LT	-	-	-	+	+	NT
5923	LT	A	IS	10.020	0.740	0.073	-	+	LT	-	-	-	-	-	-
		B	MB	10.020	0.980	0.098	+	+	ST/LT	-	-	-	+	+	NT
6014	LT	A	MB	8.850	0.580	0.066	-	+	LT	-	-	-	+	+	NT
		B	IS	6.190	0.403	0.065	-	+	LT	-	-	-	-	-	-
6108	LT	A	MB	11.760	0.790	0.067	-	+	LT	-	-	-	-	-	+
		B	IS	8.200	0.530	0.065	-	+	LT	-	-	-	-	-	-
6259	LT	A	MB	10.404	0.665	0.064	-	+	LT	-	-	-	+	+	NT
		B	IS	9.625	0.627	0.065	-	+	LT	-	-	-	-	-	-

Table 4 (continued)

Strain Number	Original Isolate Ent.[a]	Colonies On Tergitol-7		Infant Mouse Assay-ST[b]				Y-1 Adrenal Cell-LT[c]	Ent. Produced	Plate Agglutination Test					
										For K99[d]			For K88[e]		
		Number	Type	B.W.[f] gm.	G.W.[g] gm.	Ratio	Result			Minca[h]	TSA[i]	TSB[j] Minca	Minca	TSA	TSB Minca
6352	LT	A	MB	9.919	0.665	0.067	-	+	LT	-	-	-	+	+	NT
		B	IS	10.861	0.638	0.059	-	+	LT	-	-	-	-	-	-
4931	LT	A	IS	6.774	0.406	0.060	-	+	LT	-	-	-	-	-	-
		B	R	8.686	0.537	0.062	-	+	LT	-	-	-	-	-	-
		C	MB	8.157	0.525	0.064	-	+	LT	-	-	-	-	-	-
4984	LT	A	IS	8.448	0.580	0.069	-	+	LT	-	-	-	-	-	-
		B	MB	7.545	0.455	0.060	-	+	LT	-	-	-	+	+	NT
		C	IS (Brown)	7.200	0.430	0.060	-	+	LT	-	-	-	-	-	-
5592	LT	A	IR	8.658	0.463	0.053	-	-	-	-	-	-	-	-	-
		B	R	5.568	0.299	0.054	-	-	-	-	-	-	-	-	-
		C	MB	8.150	0.480	0.059	-	+	LT	-	-	-	+	+	NT
6235	LT	A	MB	10.261	0.734	0.072	-	+	LT	-	-	-	+	+	NT
		B	IS	11.561	0.837	0.072	-	-	-	-	-	-	-	-	-
		C	IR	10.775	0.704	0.065	-	-	-	-	-	-	-	-	-

PLATE I

Rough Escherichia coli colony on Tergitol-7 Agar.

Plate I

PLATE II

Escherichia coli colonies on Tergitol-7 Agar

Fig. 1. Intermediate Smooth Colony

Fig. 2. Intermediate Rough Colony

Fig. 1

Fig. 2

PLATE III

Escherichia coli colonies on Tergitol-7 Agar

Fig. 1. Mucoid A Colony (Yellow)

Fig. 2. Mucoid A Colony (Peach)

Fig. 1

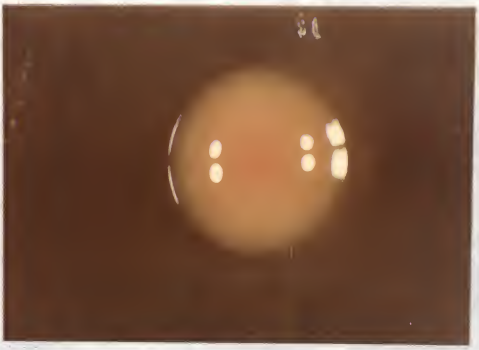

Fig. 2

PLATE IV

Escherichia coli colonies on Tergitol-7 Agar

Fig. 1. Mucoid B Colony

Fig. 2. Tetrazolium Reducing Colony

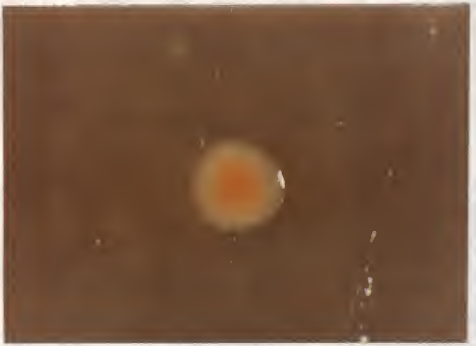

Fig. 1

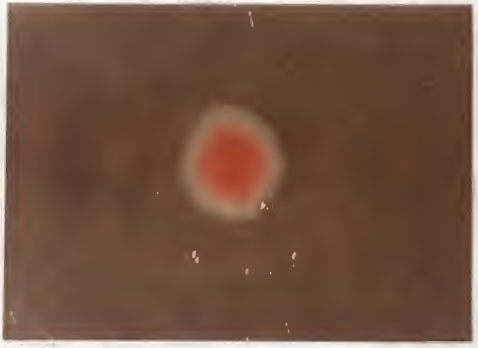

Fig. 2

COMPARISON OF ESCHERICHIA COLI VIRULENCE FACTORS WITH COLONY MORPHOLOGY ON VARIOUS MEDIA

by

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AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

Neonatal colibacillosis caused by enterotoxigenic Escherichia coli (ETEC) is of concern to livestock and public health personnel. Losses are measured not only in death, but also weight gain and medication costs.

It is accepted that ETEC cause diarrhea by colonizing the small intestine and producing enterotoxin. Virulence factors associated with colonization are appendages of bacterial cells called pili, known as K99, K88 or 987P antigens. All these colonization antigens are recognized in swine ETEC infections, and K99 and 987P are associated with infections in cattle.

Enterotoxigenic E. coli produce either heat-labile (LT) and/or heat-stable (ST) enterotoxins. Both colonization factors and enterotoxins are required for severe enteropathogenicity. After colonization, ETEC produce either ST and/or LT which induce fluid secretion into the intestinal lumen by stimulation of adenyl-cyclase activity.

Tests that have been used to identify ETEC have included production of ligated intestinal loops in live animals for ST or LT, the Y-1 adrenal cell assay for LT and the suckling mouse assay for ST. Solid phase radioimmuncassay and passive immune hemolysis have also been used to detect LT. These methods are useful but time consuming and expensive. The need for improved tests is apparent.

Colony morphology on several media was used to correlate with production of virulence factors. Clinical isolates from calves and pigs which produced either ST or LT were cultured on Tergitol-7 (T-7) medium

for isolation. Each colony type was picked and then examined for colony morphology on T-7, blood, macCoukey, congo red, minca, and minca congo red agars, retested for both types of enterotoxin production, and tested for K88 and K99.

Results confirmed usefulness of minca and passage in trypticase soy broth for detection of K88 and K99 respectively.

The production of hemolysis on blood agar by ETEC of porcine origin seemed to be associated with LT production and mucoid-B type colonies.

Three significant associations between colony type on T-7 and virulence factors were recognized. There were high correlations between mucoid-B colonies of porcine origin and production of LT and/or K88, between mucoid-A colonies of bovine origin and production of ST and/or K99, and between intermediate smooth colonies and production of LT.