THE IDENTIFICATION AND PARTIAL CHARACTERIZATION OF ENTEROTOXIN-LIKE ACTIVITY IN SALMONELLA SPECIES ISOLATED FROM HORSES

By

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ABSTRACT

THE IDENTIFICATION AND PARTIAL CHARACTERIZATION OF ENTEROTOXIN-LIKE ACTIVITY IN SALMONELLA SPECIES ISOLATED FROM HORSES

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The suckling mouse assay was used to determine the presence or absence of enterotoxin-like activity in strains of Salmonella isolated from horses. A fluid accumulating factor was demonstrated in 9 out of 17 crude culture supernates prepared from 18 h roller tube cultures of Salmonella grown in brain heart infusion (BHI) broth. Of these, 6 were Salmonella typhimurium, 2 were Salmonella anatum, and one was Salmonella cholerasuis. Enterotoxin-like activity was not stable to freezing (-70 C) and thawing, but was stable to boiling for 30 min. Maximum levels of enterotoxin-like activity occured in 8 to 14 h roller cultures of Salmonella and in 6 h shaker cultures of Salmonella in filtered BHI.

Greater than 4.0×10^6 washed cells/mouse were required to stimulate fluid secretion in the suckling mice. Enterotoxin-like activity was elicited by crude culture supernates of Salmonella containing an average of 1.9×10^6 viable cells/0.1 ml. This difference in bacterial number

and the fact that enterotoxin-like activity was retained by a Millipore filter (0.45μm) suggests that enterotoxin was primarily cell bound and was released into the growth medium in low concentration.

To Bob,

encouragement, patience and understanding

you were always there

To Joyce and Frank Ruggeri

To Donna

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INTRODUCTION

Acute diarrheal disease in both man and animals may be caused by a wide range of organisms. Several genera of enteric pathogens release enterotoxins into the lumen of the small intestine. The toxins, which act locally to stimulate fluid release and net loss of body water and electrolytes are best characterized in Vibrio cholerae. To date enterotoxins have also been demonstrated in Escherichia coli (37, 71, 86), Klebsiella pneumoniae (47, 50), Enterobacter cloacae (48), Vibrio parahaemolyticus (74), Clostridium perfringes (89), Yersinia enterocolitica (64), and Salmonella typhimurium (75, 80).

Despite the fact that Salmonella accounts for a significant amount of enteric disease, comparatively little is known about the pathogenic mechanism(s) of these organisms. Salmonella differ from most other enterobacteria in their ability to penetrate the intestinal wall and survive within cells of the reticuloendothelial system (92, 93), but invasion of mucosa alone is not sufficient to evoke fluid loss in Salmonella enteritis (28). In 1974 (73) an enterotoxin associated with Salmonella was described in isolates from human patients with gastroenteritis. Subsequently

other investigators (51, 75, 76, 79, 80) have produced, characterized, and partially purified enterotoxins from Salmonella enteriditis and S. typhimurium.

The primary objective of this study is to determine whether clinical isolates of Salmonella from horses possess enterotoxin-like activity. Salmonella isolated from asymptomatic equine carriers and from clinically active horses suffering from both acute and chronic diarrhea were evaluated. Enterotoxin activity was tested for using the suckling mouse assay (13). When a number of positive isolates were identified a second objective was to characterize selected physiological parameters of production and stability of these enterotoxin-like substances.

LITERATURE REVIEW

Bacterial enterotoxins cause diarrhea in humans and neonatal animals (calves, pigs, and lambs). The role of enterotoxins in equine diarrheal disease has not been documented. Human cholera is considered the pathophysiologic prototype of these diarrheal enterotoxic diseases. The enterotoxin responsible for fluid loss in cholera has been purified, its mechanism of action determined, and its antigenic properties studied (27, 53, 65). In reality, enteric disease produced by enterobacteria is responsible for more world-wide morbidity and mortality than cholera itself (8).

Enterotoxins of Escherichia coli

Enterotoxigenic Escherichia coli (ETEC) is the most common cause of travelers diarrhea and is a major problem among newborn infants and neonatal animals. E. coli causes disease by colonizing the small intestine and producing enterotoxins. Adherence to epithelial cell surfaces is mediated by specific heat-labile, plasmid controlled surface antigens. These antigens have been characterized as the K88 antigen of swine-specific ETEC (41), the K99 antigen of bovine- and sheep-specific ETEC (62), and the colonization

factor antigen (CFA) of ETEC isolated from man (21). The toxins (37, 71, 83), first isolated from strains enteropathogenic for pigs, have been designated as heat-labile (LT) toxins (39) and heat-stable (ST) toxins (86). Heat-labile toxin is a nondialyzable protein molecule destroyed by heating to 60 C for 30 minutes (39). Molecular weights of 20,000 (40), 100,000 (17), 200,000 (78) and greater than 300,000 daltons (52) have been reported. E. coli LT is immunogenic (39) and immunologically cross reactive with LT and cholera toxin (CT) in immunodiffusion studies (38). Antisera against either toxin will neutralize the various biological actions of both toxins (19, 82). E. coli LT stimulates adenyl cyclase activity and elevates intracellular concentrations of cyclic adenosine 3', 5'-monophosphate (cAMP) (22, 42), but on a weight basis is 100 fold less toxic than cholera toxin (71). By contrast, ST is a smaller dialyzable molecule and is stable to boiling (86). Alderete and Robertson (1) recently purified ST, a polypeptide of 47 residues. The toxin has a molecular weight of 4,400, by SDS gel electrophoresis and gel filtration, and a molecular weight of 5,133 as determined by amino acid analysis. Biological activity is not lost after treatment with pronase, trypsin, proteinase k, deoxyribonuclease, ribonuclease, phospholipase C, or acid. ST is a poor antigen (20, 83) and is weakly neutralized by anticholeragenoid (49).

The synthesis of diarrhea-inducing enterotoxins by E. coli is plasmid mediated (87). Two classes of ENT

plasmids have been recognized, one determining production of both LT and ST (37), the other determining only ST (40).

Animal and Cell Models for Detection of Enterotoxins

Enterotoxins can be readily detected in cell-free supernatant fluid following growth of bacteria in broth media. The large number of models available for enterotoxin assays were originally used to study *V. cholerae* enterotoxin and have been adapted for *E. coli*, *Salmonella* and other enteric pathogens. None are truly *in vitro* assays and all are biologically based systems. *E. coli* LT is commonly measured by the rabbit ileal loop assay (20, 84, 85), the Y-1 adrenal tumor cell culture assay (15), the Chinese hamster ovary (CHO) cell culture assay (35), and the skin vascular permeability factor assay (23). Production of ST is measured in the rabbit ileal loop assay (20) and in the infant mouse assay (13). The *Salmonella* enterotoxins, which are the major topic of this thesis, have been studied in the rabbit ileal loop assay (79, 80), the infant mouse assay (51), the CHO cell assay (76), and the skin permeability factor assay (75).

The most widely used intestinal assay is the adult rabbit ileal loop described by De and Chatterje in 1953 (12) for *V. cholerae*. In this assay the test material (either live bacteria or culture filtrates) is injected into alternating segments of the small intestine. Cholera toxin

elicits a delayed, but steadily increasing, secretory response at low as well as high doses. The volume of fluid per length of intestinal segment is recorded (ml/cm) 18 hours after injection (4). Investigators have employed various modifications of the ligated loop assay for other enterics. Evans et al. (20) showed that individual responses to E. coli ST and LT differed in a characteristic manner. Onset of net fluid accumulation in response to ST appeared between 4 and 6 hours, whereas maximum ratios elicited by LT occured between 16 and 18 hours after injection.

As with other diarrheagenic organisms, the Salmonella have been studied extensively using the rabbit model. Sakazaki (73) first demonstrated enterotoxin-like activity in Salmonella. An improved ileal loop assay was developed by Sedlock and Deibel (79). In this model a greater response rate to enterotoxin in sterile filtrates was obtained when the intestinal lumen was washed with a balanced salt solution containing a mucolytic agent (N-acetyl-L-cysteine) prior to exposure to toxin or when the toxin was given in conjunction with a non-toxin producing, invasive Salmonella. It was suggested that intestinal mucin passively acts as a barrier preventing toxin access to epithelial cells. The invasive Salmonella was thought to reduce the presence of intestinal mucin and hence eliminate a component which may act to inhibit enterotoxin action.

The infant mouse assay was originally described by Dean (13) in 1972 for E. coli ST. Four hours after

intragastric inoculation of 1 to 4 day old suckling mice the ratio of the weight of the intestinal tract and its contents to the remaining body weight (IW/BW) was determined. Ratios between 0.070 and 0.090 were scored as questionably positive while those greater than 0.090 were scored as strongly positive. This assay proved more convenient and reliable and the results paralleled data from the rabbit ileal loop model. The reproducibility and optimal growth and test conditions for the assay of ST were examined by Giannella (30). Sixteen to 24 hour roller tube cultures of E. coli grown in casamino acid yeast extract broth prove to be the optimal conditions for maximum production of ST. Samples were tested in 1 to 3 day old mice for 3 hours. A 95% confidence interval for positive IW/BW ratios was constructed with values greater than 0.083 being scored as positive, and those between 0.075 and 0.082 considered indeterminate. The day-to-day variation of the suckling mouse assay ranged from 10.5 to 15.7% depending on the bacterial strain. More recently Moon et al. (57) showed that the infant mouse assay for ST could be made more sensitive, reliable, and simpler than the standard assay by using older mice, higher temperatures of incubation, and diarrhea as the index of response. Mice older than 4 days and/or held at 37 C were exposed to ST and found to develop diarrhea. In the standard assay, 1 to 4 day mice held at 25 C, responded to ST by accumulating intestinal fluids. The alteration of the response from

fluid accumulation to diarrhea with increase in age and temperature is one reason why some mice older than 4 days or held at 37 C gave negative responses in the standard assay.

Recently cholera toxin has been shown to stimulate a positive reaction in the suckling mouse assay (91). Koupal and Deibel (51) obtained positive results with Salmonella after making slight modifications in the standard procedure.

Rabbit skin tests can be used to detect enterotoxins in E. coli (23, 84), V. cholerae (10) and Salmonella (75). In 1965, Craig (10) showed that intracutaneous injection of culture filtrates of V. cholerae caused erythema, induration and increased capillary permeability of small blood vessels in rabbit skin 18 to 24 hours after injection. Visualization of the reaction was enhanced by intravenous injection of Pontamine sky blue dye at the 18th hour. Salmonella produces a permeability factor that is indistinguishable from the reactions of CT and LT. Peterson suggests that chromatography of culture filtrates on Sephadex G-100 is required for optimal induration activity (75). Many strains of Salmonella also produce a rapid vascular permeability response with a critical bluing time of one hour. No induration is observed with the rapid permeability factor (PF) reaction. The early factor of Salmonella can be demonstrated only by administering the bluing dose within a

critical time after intradermal injection of culture filtrates. If this is not done, false positives occur or the entire reaction is blanched. Rapid PF activity is stable for at least 4 hours at 100 C, whereas the delayed PF of Salmonella, CT, and LT are heat-labile.

Various tissue culture lines have been successfully used in the assay of enterotoxin. In all systems, a sterile filtrate is added to the tissue culture medium. A logarithmically growing monolayer of cells is bathed with the suspension and the cultures are incubated. Donta et al. have shown that picogram quantities of purified cholera enterotoxin are capable of inducing morphologic changes and steroidogenesis in monolayer cultures of Y-1 mouse adrenal cells (14). Similar cellular alterations are induced by LT (15). Chinese hamster ovary (CHO) cells are also sensitive to LT and CT. These cells accumulate cAMP and elongate. The effect is apparently mediated through cellular adenyl cyclase activation (35). Changes in CHO cells indistinguishable from those produced by CT and LT are elicited by chromatographed culture filtrates of S. typhimurium (76).

Salmonellosis in Equidae

Salmonellosis represents a global problem for man and animals. Members of the genus have host-parasite relationships over a wide host range, being most prevalent in turkeys, followed by chickens, cattle and swine (58). Salmonella are also recognized as a serious and continuing

problem in horses, the world-wide prevalence ranging from 0.37 to 27% (60). Salmonellosis in Equidae is a specific diarrhea-inducing disease with significant economic implications, particularly in large equine clinic complexes.

At least forty of the recognized Salmonella serotypes are known to infect horses (60), the most prevalent being Salmonella typhimurium, Salmonella anatum, Salmonella enteriditis, and Salmonella newport (7, 60, 68). None of these are normal residents of the intestinal tract of a healthy horse (72). Salmonella-induced colitis is regarded as an important enteric infection of young foals but is often overlooked as a major cause of acute, rapidly fatal diarrhea in the adult horse (3, 7, 60, 61). Debilitated, aged and stressed horses, as well as foals, are highly vulnerable to acute salmonellosis (59).

A foal suffering from salmonellosis follows a characteristic disease pattern (7, 60, 61, 68). Initially the animal goes off feed, is depressed, weak, and its body temperature may reach 40 C or higher. Profuse, watery diarrhea developes leading to severe dehydration, electrolyte depletion, and acidosis. Blood urea nitrogen and packed cell volume values increase concurrent with leukopenia; either the neutrophil count is less than 3,600/mm^3 or a rapid decline in neutrophil number is seen (16). Unless detection and therapy are immediate, the animal's chance of survival decreases rapidly. A foal may develop

a peracute septicemic infection characterized by persistent fever, soft feces with excessive mucous, visceral absesses, and death in 24 to 72 hours. The mortality rate ranges between 17 and 50%. In the acute form the course of infection lasts from 1 to 3 weeks. The fever and diarrhea are intermittent, and frequently the foal recovers. The chronic form lasts 3 weeks to several months. The outcome is usually fatal if the foal is ill greater than 3 weeks (60). Occasionally Salmonella escape the gut and infect joints and bones (most commonly the stifle and hock joints) (7, 68).

In contrast to the foal, the normal adult horse is quite resistant to salmonellosis (68). Physical, environmental and surgical stress are important factors in the pathogenesis of adult horse illness (60). A horse with a subclinical infection may become acutely ill following transportation (9), surgery with anaesthesia (58), overtraining (58), hot, humid weather (7, 58, 60, 66), new environment, changed feeding regimen, or pregnancy. Owen (63) has also presented clinical evidence that a Salmonella carrier can develop post-stress diarrhea which may be exacerbated by tetracycline therapy that depresses or alters the normal intestine bacterial antagonists of the Salmonella. Often a similar clinical picture occurs in which Salmonella cannot be isolated from the stool. A negative rectal swab is of little value in this instance. Whether the sample is from a living or dead animal, repeated and selective measures must be undertaken to confirm a negative result.

Owen (63) suggests that Colitis X may be part of the Salmonella syndrome. Colitis X (36, 69, 90) has not been defined on the basis of an etiologic entity, but it does have recognizable characteristics similar to acute salmonellosis, i.e., history of stress, severe diarrhea, and signs of toxemia (leukopenia, fever, and death in 3 to 24 hours). Colitis X does not appear to spread laterally, whereas horizontal transmission of Salmonella is possible either by direct contact with an infected horse or by indirect contact with a contaminated environment (16).

Various therapeutic regimens for salmonellosis are employed (59, 61, 68). Initially antibiotics are administered parenterally in dosages within or higher than the recommended limits. Sensitivity is usually limited to nitrofuran products, chloramphenicol, and neomycin sulfate (61). However, specific antimicrobial therapy in peracute, many acute, and most chronic cases is generally ineffectual (60). Correcting dehydration, electrolyte imbalances, diarrhea, and shock with a balanced electrolyte solution such as lactated Ringers with $NaHCO_3$ and glucose (97) is of more value. Medication to control the diarrhea and promote appetite is of equal importance.

Salmonellosis in Humans

The pathogenicity of Salmonella in humans was first discovered by Gärtner (29) in 1888 in an epidemic of

gastroenteritis. Since then, the incidence of the disease has been on the increase (18). Modern methods of mass processing and distribution of many naturally infected foods promote the dissemination of the microbe. Furthermore, recent advances in surgery and medicine are attended by an increased risk of infection by Salmonella (6).

There are four main groups of clinical manifestations in salmonellosis that may occur individually, simultaneously or consecutively in the course of infection. They are gastroenteritis, bacteremia with or without extraintestinal localization, typhoid-like patterns, and the carrier state (81). In man, gastroenteritis is the most common clinical picture (77). Symptoms may range from mild diarrhea to a fulminant form with rapid dehydration. Usually the disease is self-limited and lasts from one to four days.

As early as 1951, McCullough (55, 56) investigated the pathogenicity of various food-born Salmonella for man. Human volunteers were fed various strains of Salmonella. All proved to be pathogenic with great variation in infectivity. The incubation period ranged from 8 to 72 hours and the cases varied in severity from mild brief enteritis to serious prostrating illness.

Increased susceptibility to Salmonella enteritis after gastric surgery has been reported (2, 95). These patients are also subject to a more severe cholera-like illness (31). The disease, however, can be differentiated

from cholera because the diarrhea is of longer duration and has a final electrolyte concentration less than that of cholera (32).

Pathogenic Mechanisms of Salmonella

The existence of a Salmonella toxin responsible for the diarrhea has been postulated for years and various investigations describe a number of possible virulence factors which may contribute to the diarrhea associated with gastroenteritis. One proposed mechanism is related to the ability of Salmonella to invade the intestinal epithelium during the course of infection.

Due to the difficulty in working with human volunteers and patients, laboratory animal models have been developed to study the pathogenesis of enteric disease. Since Rhesus monkeys develop symptoms similar to human Salmonella food infection (11), the morphological alterations of the intestinal tract have been examined in the monkey. In 1966, Kent et al. (44) discovered most severe and extensive lesions in the colon, with mild early inflammatory reaction in the ileum followed by ileal lesions. The fact that S. typhimurium was isolated from all lesions suggested that bacterial invasion of the intestinal wall was necessary for Salmonella enteritis. But unlike cholera, Salmonella diarrhea in the monkey involved both the small and large intestine.

Takeuchi and Sprinz (92, 93) showed that guinea pigs preconditioned by starvation and opium (43) developed acute enteritis following intragastric administration of S. typhimurium. By electron microscope studies they were able to demonstrate bacteria penetrating epithelial cells of the brush border into the lamina propria, where they proliferated within macrophages.

Once colonization and invasion of the intestinal lining occurs, symptoms of enteritis were manifested. In the rat Salmonella enterocolitic model (54), Powell et al. (67) showed that the secretion of water and electrolytes by the ileum was the major determinant of diarrhea in the rat. Rout et al. (70) determined that alterations in fluid transport were correlated with changes in intestinal morphology and with intestinal concentrations of Salmonella in monkeys.

Two possible mechanisms could account for the net transport of fluid and electrolytes into the bowel lumen; (1) passive transudation of fluid or (2) active electrolyte secretion. That certain invasive strains of Salmonella did alter active transport across ileum was determined utilizing the adult rabbit intestinal loop model (28). Taylor and Wilkins (94) first demonstrated that live, invasive cultures of Salmonella would cause dilation and fluid accumulation in ligated rabbit intestinal loops. The relationship of mucosal invasion to the mucosal inflammatory

response and fluid production by the rabbit ileum was investigated by Giannella et al. (33). Mucosal invasion is essential to the pathogenesis of salmonellosis but extensive mucosal inflammation did not seem to be a necessary prerequisite or directly responsible for fluid secretion. Upon examining ion transport across isolated ileal mucosa (28), invasive Salmonella were found to inhibit active sodium absorption and stimulate active chloride secretion. Since the addition of cAMP (25) or cholera toxin (26) to normal ileal mucosa enhances these same secretory processes, increased levels of adenyl cyclase were looked for. Giannella (34) showed that S. typhimurium infection resulted in activation of mucosal adenyl cyclase and that the accumulation of cAMP concommittant with ileal fluid secretion suggests that the adenyl cyclase-cAMP system mediates the secretion. Although a bacterial property or factor, in addition to invasion and inflammation of the gastrointestinal mucosa seems to be responsible for fluid exorption, positive loops could not be induced by culture filtrates (33), suggesting enterotoxin was not responsible for fluid loss.

Salmonella infection of the intestine and the attendant acute inflammatory process may result in the local synthesis of prostaglandins which activate mucosal adenyl cyclase activity (45). Elsewhere in the gastrointestinal tract, acute inflammation results in synthesis

and release of prostaglandins (96), which can then stimulate intestinal cyclase activity (45). Indomethacin (88) (a potent inhibitor of prostaglandin synthesis) abolishes Salmonella-mediated adenyl cyclase activation and fluid secretion (34), but only partially inhibits cholera toxin-mediated secretion without altering the activation of adenyl cyclase (46). This difference suggests the involvement of prostaglandins in the Salmonella-mediated but not cholera toxin-mediated activation of adenyl cyclase.

The ability of Salmonella to produce enterotoxic factors in culture filtrates was first demonstrated by Sakazaki et al. (73) in the rabbit intestinal loop model. Eleven out of 13 Salmonella filtrates gave positive loop reactions. Histological examinations of sections of the intestinal mucosa from positive loops showed pathological changes similar to those produced by toxigenic E. coli.

Evidence for a protein enterotoxin was reported by Koupal and Deibel (51, 80). The toxin, most of which is strongly bound in the outer membrane of the cell wall of S. enteriditis, was obtained from the organism in its exponential phase of growth in brain heart infusion (BHI) broth shaker culture. The activity of the enterotoxin (determined by the suckling mouse model) was destroyed by pronase, but was resistant to α amylase, trypsin, lysozyme, phospholipase A, C, or D, acidity, and alkalinity. Utilizing the rabbit ileal loop assay, Koupal and Deibel (80)

also detected enterotoxin activity from S. typhimurium grown in various complex and defined media. Activity was observed in culture filtrates of the organism in mid stationary growth in BHI, 2% Casamino Acids media, synthetic amino acids medium, and a minimal glucose-salts medium. The toxin appears stable to low temperatures but a decrease in activity is observed when the preparations are frozen and thawed. Attempts at molecular weight characterization were inconclusive.

An additional factor that might be involved in Salmonella gastroenteritis has been described by Sandefur and Peterson (75, 76). Initially characterized as a delayed permeability factor, it proved to be heat-labile, causing erythema and induration after 18 hours in a rabbit skin test, and effecting the elongation of Chinese hamster ovary cells. The culture filtrate, containing this factor, must be passed through a Sephadex G-100 column in order to remove an inhibitor-like substance that was found to mask factor activity. Even though intestinal secretory activity was not demonstrated, it appears similar to cholera enterotoxin since it can be neutralized by cholera antitoxin and therefore may play an active role in Salmonella gastroenteritis.

The biological properties of the substances produced by Salmonella (i.e., skin permeability alteration, CHO cell elongation, rabbit intestinal and suckling mouse fluid accumulation) are shared by both cholera and E. coli

enterotoxins. Even though evidence suggests they may be responsible for fluid and electrolyte loss during intestinal infections with V. cholerae and E. coli, the actual pathogenic role of these substances is unknown in human and equine salmonellosis.

MATERIALS AND METHODS

Cultures

The Salmonella typhimurium (case numbers 3CH, 22CH, 875H, 18CH, 20CH, 844H, T24CH and E9288), Salmonella anatum (case numbers 25CH, 2CH, 166H, 017H, E312,153, 200H, 184H and A24CH) and Salmonella cholerasuis NADL used in this study were obtained from Dr. E. V. Morse, School of Veterinary Medicine, Purdue University, Lafayette, Indiana. All organisms were isolated from asymptomatic equine carriers and from clinically active horses suffering from both acute and chronic diarrhea. The clinical histories of some but not all patients were available and are published (60).

In certain phases of the study, a toxigenic E. coli strain, obtained from Dr. J. W. Peterson, University of Texas, Galveston, was included for control purposes.

Media and Maintenance of Cultures

The original cultures of Salmonella were maintained on tryptose agar slants (Difco Laboratories, Detroit, Michigan) at 4 C. For storage, all Salmonella were grown in 10 ml of BHI broth (Difco Laboratories, Detroit, Michigan), pH 6.8 in stationary culture. After 24 h at 37 C, the cultures were centrifuged at 7,710 x g for 20 min on a

Sorvall RC-5 Superspeed Refrigerated Centrifuge (Ivan Sorvall Inc., Norwalk, Connecticut). The sedimented cells were washed twice in 0.85% NaCl, resuspended in 5 ml of physiological saline, added to 5 ml of glycerol (Mallinckrodt, St. Louis, Missouri) and frozen at -20 C. Organisms treated in this manner served as the source of inocula for all cultures.

Brain heart infusion (BHI), filtered BHI, or Evan's casamino acid yeast extract (CA-YE) broth (19) were used as growth media. The CA-YE contained 20 g Casamino Acids (Difco Laboratories, Detroit, Michigan), 6 g yeast extract (Difco Laboratories, Detroit, Michigan), 2.5 g NaCl, 8.71 g 0.05 M $K_2HPO_4 \cdot H_2O$, and 1 ml of trace salts (5% $MgSO_4 \cdot 7H_2O$, 0.5% $MnCl_2 \cdot 4H_2O$, and 0.5% $FeCl_2$ dissolved in 0.001 N H_2SO_4). Volume was brought to 1 liter and pH adjusted to 8.5 with 0.5 N NaOH. When appropriate BHI was filtered with XM300 membrane (Amicon, Lexington, Massachusetts) and pH adjusted to 7.85 with 0.5 N NaOH.

Serial tenfold dilutions of culture filtrates were plated on tryptose agar for bacterial count determination.

Preparation of Enterotoxin Samples

Enterotoxin material was prepared by inoculating broth with glycerol storage cultures. Usually 5 ml of broth in 150 x 16 mm tubes was inoculated with 1 drop of cells and incubated at 37 C in a Wheaton Roller Culture Apparatus (Wheaton Scientific, Millville, New Jersey), speed setting

1/2 rev/min. After 18 h the cultures were centrifuged at 12,100 x g for 20 min on a Sorvall RC-5 Superspeed Refrigerated Centrifuge. The supernatant fluids from the broths were used as the crude preparations for inoculation of mice, or were filtered through a Swinnex-13 membrane filter (Millipore Corp., Bedford, Massachusetts) with a pore size of 0.45 μm before testing. Since previous studies (30) suggested that filtered and unfiltered supernatant fluids behave identically in suckling mice, the bulk of studies were done with unfiltered supernatant fluids.

For comparative growth studies, overnight cultures of Salmonella were also inoculated into filtered BHI, the pH adjusted to 7.85, optical density 0.060 at 520 nm, and incubated at 37 C in a Gyrotory shaker water bath (New Brunswick Scientific, New Brunswick, New Jersey), 130 rev/min, for various periods of time and enterotoxin material was prepared.

Effect of Heat on Enterotoxic Activity

Sealed portions (2ml) of the test sample were heated at 100 C in a water bath for 30 min. After heating, the samples were cooled to 37 C and assayed.

Chemicals

Enterotoxin preparations were diluted in 0.01 M Tris (hydroxymethyl) aminomethane buffer (Sigma, St. Louis, Missouri), pH 8.0.

One hundred mg of Salmonella typhimurium W lipopolysaccharide (Difco Laboratories, Detroit, Michigan) was resuspended in 10 ml of 0.85% saline. The total volume was split and dispensed into 10 separate vials and stored at -5 C until assayed for enterotoxin activity. Twofold dilutions of LPS were made using physiological saline as the diluent.

Mice

Breeding colonies of two month old 18 to 20 g HA/ICR mice (Spartan Research Animals, Haslett, Michigan) were established and their litters, approximately 10 to 16 mice per litter, were used in the suckling mouse assay. The mice were housed 6 per cage, with pine wood chips as bedding in a room at 24 C. Food (Wayne Lab-Blox, Allied Mills Inc., Chicago, Illinois) and water were available ad libitum.

Suckling Mouse Assay

The suckling mouse assay described by Dean et al. (13) was modified as follows. Newborn suckling mice (2 to 5 days old) were separated from their mothers immediately before use and randomly divided into groups of three. Each mouse was inoculated (intragastric, percutaneous injection with a 30 1/2 gauge needle) with 0.1 ml of a crude culture filtrate or control material containing 2 drops of 2% Evans Blue dye/ml. After exposure, mice were placed in boxes divided into compartments (6 x 10 x 6 cm deep) and kept at

room temperature. Two and 1/2 h after inoculation the mice were killed by cervical dislocation, the abdomen opened, and the entire intestine (not including the stomach) removed with a hemostat. The intestine from each mouse was weighed. The ratio of gut weight to remaining carcass weight was calculated and an average value was obtained. Each experimental sample was assayed using three mice and each experiment was at least done in duplicate. Activity in the assay is expressed as the ratio of intestinal weight/body weight (IW/BW). Values greater than or equal to 0.084 were considered positive, values less than 0.075 negative and those between 0.075 and 0.084 indeterminate. These values are comparable to those reported by other investigators (30, 51).

Statistics

The mean of IW/BW ratios $\pm$ 1 standard error of the mean for each sample tested was calculated (5).

RESULTS

Production of Enterotoxin-Like Activity

Seventeen *Salmonella* isolates from horses (Table 1) were examined for enterotoxin-like activity in the suckling mouse assay. Cultures grown for 18 h in BHI broth in roller tubes were centrifuged at 12,100 x g for 20 min. Non-sterile supernates were assayed immediately after harvesting cells (Table 2). A positive response (IW/BW $\geq$ 0.084) of fluid accumulation was found in 9 out of the 17 isolates. Of these, 6 were *Salmonella typhimurium*, 2 were *Salmonella anatum*, and one was *Salmonella cholerasuis*. Only one strain, *Salmonella anatum* 2CH, was clearly negative. The remaining 7 isolates were indeterminate (0.075 to 0.084) by IW/BW ratios. Some physiological parameters of this toxin activity and its production are described below. In subsequent tests, only positive strains 200H, 20CH, T24CH, E9288 and NADL and negative strain 2CH were used.

Temperature Stability of Enterotoxin-Like Activity

Culture supernates of the positive *Salmonella* species were frozen at -70 C not less than 1 or more than 6 weeks.

Table 1.--*Salmonella* Species and Source of Isolation.

Case Number	Species	Source
25CH	*S. anatum*	Asymptomatic carrier
2CH	*S. anatum*	Asymptomatic carrier
3CH	*S. typhimurium*	Asymptomatic carrier
22CH	*S. typhimurium*	Asymptomatic carrier
875H	*S. typhimurium*	Asymptomatic carrier, stress
18CH	*S. typhimurium*	Asymptomatic carrier, stress
166H	*S. anatum*	Stress, intermittent diarrhea
017H	*S. anatum*	Stress, diarrhea, septicemia
E312,153	*S. anatum*	Stress, acute diarrhea
20CH	*S. typhimurium*	Stress, acute diarrhea, foal
200H	*S. anatum*	Stress, acute diarrhea
844H	*S. typhimurium*	Stress, severe diarrhea, septicemia
184H	*S. anatum*	Chronic diarrhea
A24CH	*S. anatum*	Stress, chronic diarrhea, fatal
T24CH	*S. typhimurium*	Stress, chronic diarrhea, fatal
E9288	*S. typhimurium*	Fatal
NADL	*S. cholerasuis*	Unknown

Table 2.--Production of Enterotoxin-like Activity in BHI Roller Tube Cultures of *Salmonella*[a].

Enterotoxin Activity	Case Number	IW/BW Ratio
Negative	Broth Control	.072 ± .003 (10)
	2CH	.071 ± .003 (5)
Indeterminate	A24CH	.075 ± .002 (3)
	3CH	.077 ± .003 (2)
	E312,153	.077 ± .004 (2)
	25CH	.078 ± .006 (2)
	22CH	.078 ± .002[b]
	017H	.080 ± .003 (3)
	166H	.083 ± .002 (4)
Positive	875H	.084 ± .005 (5)
	200H	.086 ± .001 (4)
	184H	.089 ± .007 (2)
	20CH	.090 ± .006 (3)
	T24CH	.092 ± .005 (6)
	844H	.093 ± .005 (3)
	E9288	.097 ± .002 (7)
	18CH	.100 ± .004 (2)
	NADL	.100 ± .005 (5)

[a]Figures represent mean ± 1 standard error of the mean of at least 2 separate assays of toxin preparations tested immediately upon harvesting filtrates. Numbers in parentheses indicate the number of tests. Each test used 3 mice.

[b]Figure represents mean ± 1 standard deviation.

Thawed preparations were tested for toxin activity in the infant mouse assay. Freeze-thaw ratios over the entire storage period were equivalent and consequently the data has been grouped. The data (Table 3) show that Salmonella enterotoxin-like activity is not stable to freezing.

Roller culture supernates of 3 Salmonella strains possessing enterotoxin-like activity were subjected to 100 C for 30 min. The toxic factor was stable to heat (Table 4).

Production of Enterotoxin-Like Activity Under Various Conditions of Bacterial Growth

An overnight culture of S. typhimurium E9288 was inoculated into 70 ml of filtered BHI (initial O. D. 0.060, 520 nm) and incubated in a Gyrotory shaker water bath at 37 C. The storage culture of E9288 was also inoculated into 5 ml of BHI and incubated in a roller tube apparatus at 37 C. Growth curves were plotted for both cultures (Figure 1). Shaker cultures of E9288 entered logarithmic phase of growth immediately, whereas a lag in cell division occured in roller cultures. By the 6th to 8th h, both cultures reached the stationary phase, with final cell number greater in the shaker culture.

Culture supernatant fluids from E9288 were titrated for toxin activity during various stages of growth in shaker and roller tube cultures. The relative toxin level in the culture supernates was determined by use of serial twofold dilutions of the toxin in Tris buffer. Experimentally, at

Table 3.--Loss of Enterotoxin-like Activity After a 6 Week Period of Storage at -70 C[a].

Case Number	IW/BW Ratio	
	Before	After
200H	.086 ± .001 (4)	.069 ± .002 (3)
20CH	.090 ± .006 (3)	.080 ± .003 (3)
T24CH	.092 ± .005 (6)	.073 ± .007 (2)
E9288	.097 ± .002 (7)	.080 ± .004 (3)
NADL	.100 ± .005 (5)	.081 ± .003 (3)
E. coli[b]	NT[c]	.100 ± .010 (2)
Broth Control	.072 ± .003 (10)	.070 ± .006 (3)

[a]Figures represent mean ± 1 standard error of the mean of at least 2 separate assays of toxin preparations grown in BHI in roller tubes for 18 h. Numbers in parentheses indicate the number of tests. Each test used 3 mice.

[b]*E. coli* grown in CA-YE and BHI broth in roller tubes for 18 h.

[c]Not tested.

Table 4.--Retention of Enterotoxin-like Activity after Heat Treatment[a].

Case Number	IW/BW Ratio	
	No Heat	100 C, 30 min
20CH	.084 ± .010 (2)	.088 ± .006 (3)
E9288	.096 ± .006 (3)	.094 ± .002 (5)
844H[b]	.098 ± .003	.102 ± .007
Broth Control	.074 ± .003 (5)	.078 ± .004 (2)

[a]Figures represent mean ± 1 standard error of the mean of at least 2 separate assays of toxin preparations grown in 5 ml BHI broth in roller tubes. Numbers in parentheses indicate the number of tests. Each test used 3 mice.

[b]Figures represent mean ± 1 standard deviation.

Figure 1. Growth Curve of S. typhimurium E9288 in Roller Culture (●) and Shaker Culture (▲).

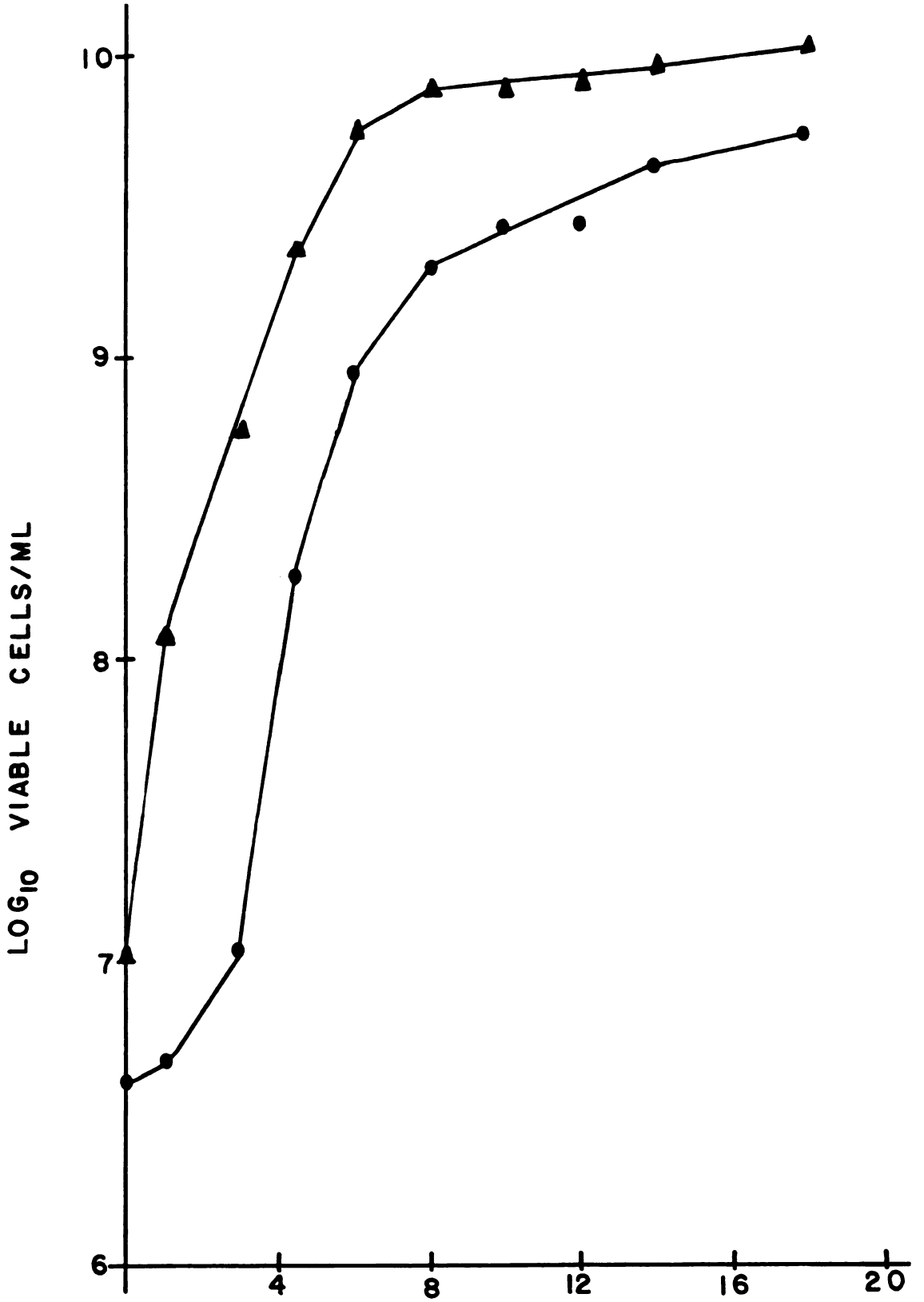

FIGURE 1

designated intervals, 5 ml of the shaker culture were removed or a roller tube culture was harvested. The last dilution giving an IW/BW ratio greater than or equal to 0.084 was considered positive and was plotted against time (Figure 2). At least 2 separate assays of toxin preparations were tested at each time point for each dilution. Maximum enterotoxin-like activity was observed in culture supernates prepared from 8 to 14 h roller cultures and from 6 h shaker cultures. No enterotoxin-like activity could be demonstrated in 18 h shaker cultures compared to 18 h roller cultures of Salmonella.

Loss of Enterotoxin-Like Activity Upon Filter Sterilization of Samples

One ml of crude culture supernate prepared from 18 h roller tube cultures, of toxigenic E9288 and non-toxigenic 2CH, was filtered through a Millipore membrane. The sterile filtrate was examined for enterotoxin-like activity in suckling mice. The used Millipore filter was resuspended in 1 ml of BHI and tested for toxin activity (Table 5). Salmonella typhimurium E9288 preparations lost their enterotoxin-like activity upon filtration. The factor responsible for the activity observed in the mice was retained by the Millipore filter. Both the sterile filtrate of Salmonella anatum 2CH and the membrane used to filter 2CH failed to exhibit enterotoxin-like activity. This strain was also negative in the unfiltered preparations (Table 2 and 5).

Figure 2. Titration of S. typhimurium E9288 Enterotoxin-like Activity in Roller Culture (●) and Shaker Culture (▲).

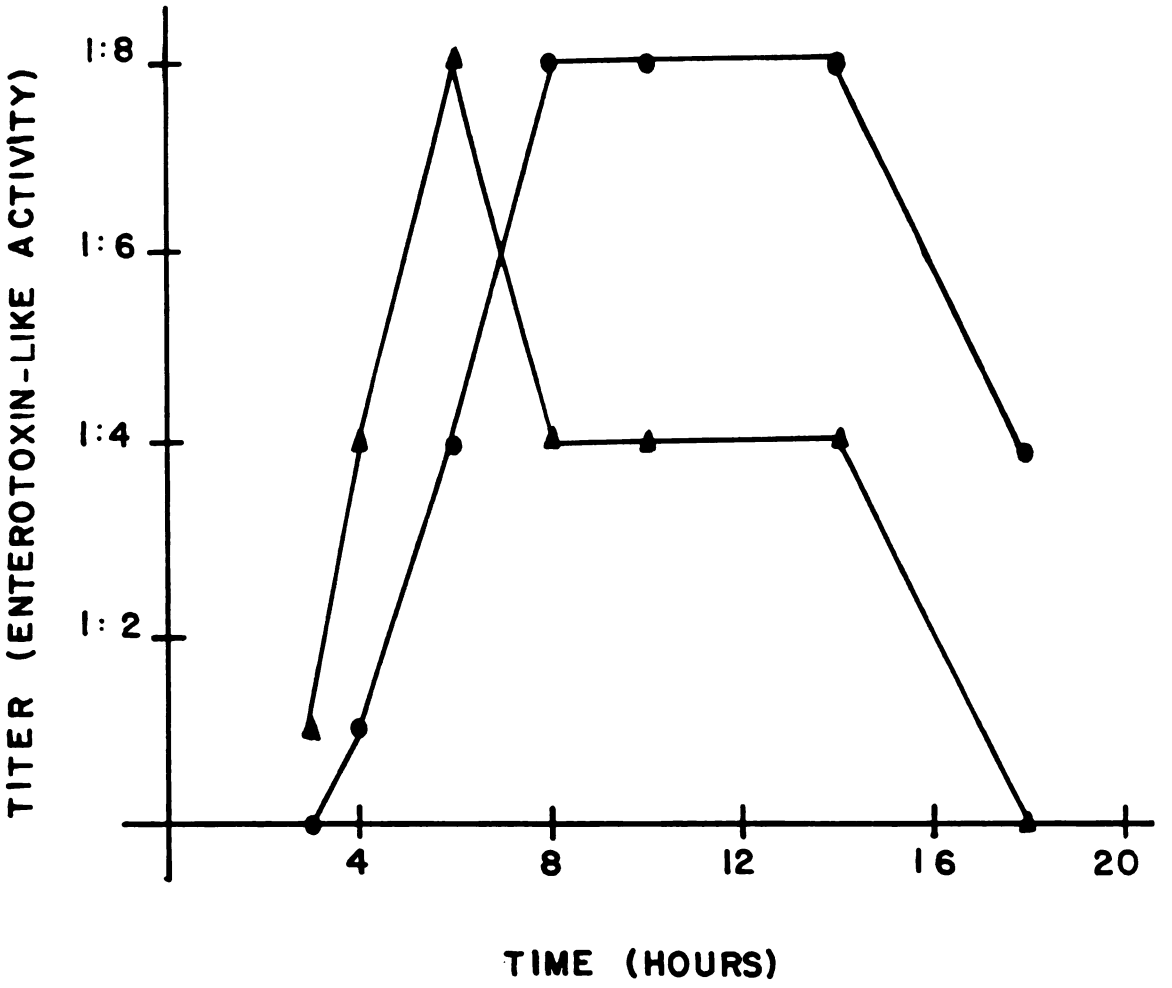
TITER (ENTEROTOXIN-LIKE ACTIVITY)
1:8
1:6
1:4
1:2
4
8
12
16
20
TIME (HOURS)

FIGURE 2

Table 5.--Loss of Enterotoxin-like Activity After Filter Sterilization of Supernatant Fluids[a].

Case Number	Crude Filtrate		Sterile Filtrate
	IW/BW Ratio	Count[b]	IW/BW Ratio
E9288	.097 ± .002 (7)	1.9×10^6	.071 ± .002 (7)
filter[c] E9288	.097 ± .004 (3)		
2CH	.071 ± .003 (5)	3.2×10^4	.064 ± .000 (2)
filter[c] 2CH	.070 ± .001 (2)		

[a]Figures represent mean ± 1 standard error of the mean of at least 2 separate assays of toxin preparations from 18 h roller tube cultures. Numbers in parentheses indicate the number of tests. Each test used 3 mice.

[b]Average number viable Salmonella/0.1 ml supernatant fluids.

[c]Millipore filter (used to sterilize 1.5 ml of supernate) resuspended in 1 ml of BHI.

Activity of Lipopolysaccharide (LPS) in the Suckling Mouse Assay

The possibility of LPS being associated with enterotoxin-like activity in the culture supernatant fluids of Salmonella led to testing of commercially prepared LPS in the infant mouse assay. Doses up to 1 mg/mouse failed to enhance fluid secretion (Table 6).

Table 6.--Inability of Salmonella LPS to Stimulate Enterotoxin-like Activity in Suckling Mice[a].

Dose (µg)	IW/BW Ratio
62.75	.077 ± .002 (3)
125.00	.073 ± .002 (3)
250.00	.079 ± .002 (3)
500.00	.073 ± .001 (3)
1000.00	.076 ± .003 (3)
0.85% NaCl	.067 ± .001 (5)

[a]Figures represent mean ± 1 standard error of the mean of at least 2 separate assays of LPS resuspended in 0.85% saline. Numbers in parentheses indicate the number of tests. Each test used 3 mice.

Activity of Live Salmonella in the Suckling Mouse Assay

An investigation of the number of viable organisms remaining in the enterotoxin preparations was undertaken. Bacterial counts were determined for 18 h roller tube culture supernates. Salmonella typhimurium E9288 had an

average of 1.9×10^7 viable cells/ml of supernate. Salmonella anatum 2CH had an average of 3.2×10^5/ml of supernate (Table 5).

The sedimented cells from E9288 and 2CH were washed twice in physiological saline and resuspended in 5 ml of saline. Salmonella were diluted and the resulting cell suspensions were examined for enterotoxin-like activity in suckling mice (Table 7). Greater than 4.0×10^6 S. typhimurium E9288/mouse were necessary to stimulate intestinal secretion. Salmonella anatum 2CH were unable to elicit a positive response in the mice, even at doses as high as 1.8×10^8 bacteria per mouse.

Table 7.--Reactivity of Suckling Mice to Washed Cells of *Salmonella*[a].

Dose[b]	IW/BW Ratio
	E9288
$2.5 \times 10^8 - 7.8 \times 10^8$	.093 ± .002 (4)
$1.1 \times 10^7 - 7.8 \times 10^7$	.091 ± .003 (5)
$1.1 \times 10^6 - 4.0 \times 10^6$	.067 ± .006 (3)
$1.1 \times 10^2 - 1.1 \times 10^5$	.063 ± .001 (4)
	2CH
1.8×10^8	.067 ± .009[c]
$1.0 \times 10^7 - 6.0 \times 10^7$	.072 ± .002 (3)
$1.0 \times 10^6 - 6.0 \times 10^6$	.063 ± .002 (3)
$1.0 \times 10^5 - 6.0 \times 10^5$	.065 ± .002 (2)

[a]Figures represent mean ± 1 standard error of the mean of at least 2 separate assays of washed cell preparations. Numbers in parentheses indicate the number of tests. Each test used 3 mice.

[b]Dose range recorded as the number of bacteria injected per mouse.

[c]Figure represent mean ± 1 standard deviation.

DISCUSSION

Swine, calves and lambs suffer from an acute diarrheal disease brought on by toxigenic E. coli. Horses experience a similar clinical syndrome, frequently associated with Salmonella, but enterotoxin-like activity has never been associated with equine salmonellosis. Using strains of human origin, Koupal and Deibel (51) have shown that the suckling mouse assay is sensitive to Salmonella enterotoxin. This assay was chosen to survey for toxin activity in the 8 strains of S. typhimurium, 8 strains of S. anatum and one strain of S. cholerasuis. For E. coli, 16 to 24 h roller tube cultures in CA-YE is optimal for toxin production (30). In this study BHI broth was used since most investigators find this medium best for screening Salmonella for enterotoxin (51, 75).

In the present study, 9 strains were shown to produce enterotoxin-like activity in the suckling mouse assay. Six (875H, 18CH, 844H, 20CH, T24CH, and E9288) out of the 8 S. typhimurium cultures gave positive responses. Of these, 4 were from diarrheal cases and 2 were from carriers. Two (200H and 184H) out of the 8 S. anatum cultures gave positive responses, both isolated from diarrheal cases. The

S. cholerasuis also gave a positive response. No case history was available. Enterotoxin-like activity was not demonstrated in 8 cultures. Half were from carriers and the other half from diarrheal cases. It appears that no direct correlation between the ability of Salmonella to produce an enterotoxin-like reaction and severity of the disease state can be made.

Knowledge of the effects of various storage conditions on enterotoxin activity is critical because suckling mice are often not available when the samples are prepared. E. coli ST is stable at -20 C for up to 6 months (30) and is stable at -70 C up to 3 weeks (13). According to Koupal and Deibel (51), S. enteriditis toxin is stable at 4 C, but progressively less stable when frozen at -20 C. In this study, toxigenic supernates were stored at -70 C and assayed for activity over the following 6 weeks. Enterotoxin-like activity could not be demonstrated suggesting that toxin activity was not stable to freezing. The toxicity of cholera enterotoxin is also lost after freezing (Dr. G. Yang, 1978, personal communication).

The enterotoxins of E. coli are differentiated primarily by their reactivity after heating. The heat-stable toxin is stable at 100 C but is destroyed by autoclaving (86). The heat-labile toxin is inactivated by heating to 60 C for 30 min (39). Sandefur and Peterson (75) using Salmonella observed that the rapid permeability factor in

rabbit skin tests was heat-stable (100 C) up to 4 hours and the delayed permeability factor was heat-labile (75 C, 30 min). Infant mouse assays were not performed by these investigators. Koupal and Deibel (51) characterized an enterotoxic factor in suckling mice. Activity was lost at 80 C. Heat reactivity of the toxin-like activity of Salmonella was examined in this study. Culture supernates of 3 different Salmonella were exposed to 100 C for 30 min. Positive IW/BW ratios were obtained indicating that the toxins examined were heat-stable. Since vegetative cells in the supernatant fluids are killed within minutes by exposure to boiling water, something other than live Salmonella must have been responsible for the enterotoxin-like activity observed after this treatment.

Sedlock et al. (80), using the rabbit ileal loop assay, noted that not all media exhibited an equivalent ability to support production of Salmonella enterotoxin. Likewise, variability with regard to production of rapid permeability factor produced by Salmonella in different media was observed by Sandefur and Peterson (75). Reproducible results were only obtained when exponential phase cultures of Salmonella were tested in suckling mice (51). Giannella noted similar effects with E. coli in infant mice (30). The dependence of enterotoxin-like activity on cultural conditions of Salmonella was, therefore, examined. In this study, enterotoxin-like activity in

roller culture tubes was detected by the 4th hour of growth. The appearance of activity may depend on cultures reaching a critical cell number (1.9 x 10^8/ml at 4th hour) before toxin can be detected. Enterotoxin-like activity peaked in the stationary phase of growth (8 to 14th hour) where one log increase in cell number was noted (Figure 1). Maximum levels of toxin were detected sooner in shaker culture (6 h), but it dropped at a much faster rate than in roller culture. Activity was no longer detectable at 18 h. Sandefur and Peterson (75) discovered an inhibitor-like substance in culture supernatants which masked the effects of the delayed permeability factor in rabbit skin tests. Whether or not a similar inhibitor exists in this system is not known.

Some confusion exists in the literature regarding the need for presence of viable organisms for successful reactivity of enterotoxin assays. Giannella (30) reported that filtered and unfiltered preparations of E. coli enterotoxin react identically. Sedlock and Deibel have shown that sterile culture filtrates of Salmonella do not consistently stimulate fluid secretion in rabbit loops (79) but that reproducible results can be obtained if invasive, nontoxin producing Salmonella are injected concurrent with sterile toxin-containing preparations. They suggest the invasive process reduces the presence of intestinal mucin which physically inhibits the enterotoxin from reaching its

target cells. In this study 2 to 5 ml of Salmonella preparations were filtered through a 0.45 μm membrane 13 mm in diameter. The filters clogged rapidly. Enterotoxin-like activity could not be demonstrated in the sterile filtrates. That the filter did hold back the fluid accumulating principle was shown when positive IW/BW ratios were obtained with the suspension made from the membrane. Viable Salmonella are present on the filter. Giannella et al. (33) have presented substantial evidence which supports the concept that penetration of the epithelial surface by the bacteria is a necessary step in the pathogenesis of infection. Their studies indicate that strains lacking this ability do not cause fluid accumulation in rabbit loops. The possibility exists that invasion may play a role in stimulating enterotoxin-like activity in the suckling mice.

To determine whether there was any correlation between the number of viable bacteria/ml of supernatant fluid and the ability to promote fluid accumulation, bacterial counts were made. E9288, a strain possessing enterotoxin-like activity, had an average of 1.9×10^7 viable cells/ml of supernate. 2CH, a strain never demonstrating activity, had an average of 3.2×10^5/ml. The 2 log difference was not responsible for the differences in reactivity since as many as 1.8×10^8 S. anatum 2CH were not able to elicit fluid accumulation. Hence, cell number alone is not responsible for the enterotoxin-like activity

observed herein. Even when the membrane used to sterilize 2CH was resuspended in BHI and assayed in the mice, activity was not observed. An apparent difference exists between S. anatum 2CH and S. typhimurium E9288.

Crude toxin preparations of E9288 (an average of 1.9×10^6 bacteria/0.1 ml) stimulated fluid secretion. When up to 4×10^6 washed E9288 were injected per mouse, fluid accumulation was not observed. Higher doses of Salmonella E9288 did, however, induce positive IW/BW ratios. Koupal and Deibel (51) determined that the active enterotoxin material in Salmonella originates from the outer membrane of Salmonella and that it is released during growth. The difference in bacterial number and the fact that enterotoxin-like activity was retained by Millipore filters suggest that enterotoxin is primarily cell bound and is released into the growth medium in low concentrations.

Since endotoxin is present in all 17 preparations and only 9 gave positive responses in the mouse assay, a causal relationship for LPS in diarrhea production could be tentatively eliminated. To confirm this assumption further, commercially prepared endotoxin was tested in infant mice. Negative responses were observed with up to 1 mg of LPS. Previous investigations with E. coli and Salmonella endotoxin have also demonstrated that LPS does not provoke positive responses (39, 51, 86).

Salmonella multiplication and the presence of live bacteria in the intestine appears essential for the

development of the diarrheal syndrome. The precise role of enterotoxin needs additional characterization but the present study clearly supports the notion that toxins distinct from lipopolysaccharide exist in Salmonella and conceivably play a role in enteric disease.

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