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Probing for Enterotoxigenicity Among the Salmonellae: an Evaluation of Biological Assays

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Sixty-eight *Salmonella* strains representing 39 serotypes were variously screened for enterotoxigenicity by using the Chinese hamster ovary (CHO), Y1 adrenal, and Vero cell tests, rabbit skin tests for delayed permeability factor (DPF) and rapid permeability factor (RPF), the rabbit ileal loop test, and the infant mouse test. An iron-sufficient medium, TY-1, and a deferrated medium, DF, were compared. Of the culture supernatant fluids of strains grown in DF medium, 66% yielded positive reactions in the CHO cell test compared with only 10% with TY-1 medium. The corresponding performances with supernatant fluids of DF medium cultures in Y1 adrenal and Vero cell tests were 85 and 69% positive, respectively. The overall agreement between the Y1 adrenal or CHO cell test and the rabbit skin test for DPF, i.e., positive or negative in both tests, was about 70%. Positivity in DPF tests was a better predictor of positivity in either the Y1 adrenal or rabbit ileal loop test than vice versa. CHO cell, DPF, and rabbit ileal loop reactivities of unheated culture filtrates were each neutralized by anticholera antitoxin. Only four strains gave positive reactions in the infant mouse test, whereas up to 66% were positive for RPF in rabbit skin, based on positivity in TY-1 or DF medium or both. DPF and RPF were produced by 35% of the strains. Of the 28 isolates from human stools, 82 and 92% and all of 11 strains tested were positive in the DPF, Y1 adrenal cell, and rabbit ileal loop tests, respectively. The corresponding data for 17 sewage isolates, representing 17 different serotypes rarely isolated from human stools in Sweden, were 63 and 69% and 8 of 8 tested. On the basis of this investigation, rabbit skin tests for both DPF and RPF provide the most reliable means of screening for enterotoxigenicity among salmonellae.

Salmonellae have a particularly wide distribution in nature, encompassing both man and animals and their environments (2, 19, 36, 39). Moreover, their role in gastroenteritis and food poisoning cannot be overemphasized (26, 38, 39). Invasive properties among salmonellae are widely recognized (see references 27, 40). However, production of enterotoxins by salmonellae, as elaborated by *Vibrio cholerae* and *Escherichia coli,* is a more recent observation. Although Taylor and Wilkins (37) demonstrated dilatation of rabbit ileal loops injected intraluminally with live salmonellae, Sakazaki et al. (28) first showed positive rabbit gut dilatations with cell-free culture filtrates. Histopathology of the loops revealed changes not dissimilar to those effected by enterotoxigenic *E. coli.*

Production, assay, and characterization of salmonellae enterotoxins have been studied principally with *Salmonella enteritidis* and *S. typhimurium* (21, 25, 29, 30, 32, 33). Both heat-stable and heat-labile factors have been described. Positive infant mouse tests (9, 21) and a rapid rabbit skin test are elicited by heat-stable factor(s) (25, 29). A heat-labile toxin gives rise to a delayed skin permeability reaction with induration and causes elongation of Chinese hamster ovary (CHO) cells (29, 30).

Despite the limited surveys of Sedlock and Deibel (32) and of Sakazaki et al. (28) and a more comprehensive prospective analysis for enterotoxigenicity among salmonellae (22), there is a need for a wider knowledge of the occurrence of enterotoxigenicity within the genus *Salmonella* for evaluation of the possible role of such an enterotoxic factor(s) in the pathogenesis of *Salmonella* infections. In this study, a representative group of *Salmonella* serotypes isolated from animal, human, and environmental sources in Sweden was investigated by utilizing both the rapid and delayed rabbit skin models (29), the rabbit ileal loop and infant mouse tests (21, 32), and three widely used cell tests, namely, Y1 adrenal, CHO, and Vero cell tests (10, 15, 30, 34),

to assess production of enterotoxic factors and to characterize plausible biological patterns of enterotoxigenicity among the salmonellae. The comparative efficacies of the biological assays were also evaluated.

A relationship between production of bacterial toxins and the concentration of iron in growth media has been noted in numerous studies (3, 4, and references therein). The yields of several extracellular proteins produced by *Pseudomonas aeruginosa* have been shown to be influenced by the concentration of iron in the growth medium, with low concentrations increasing yields (3, 4). Accordingly, an iron-containing medium and a deferrated medium were evaluated for enterotoxin production by salmonellae.

MATERIALS AND METHODS

***Salmonella* strains.** A total of 68 animal, human, and environmental isolates of salmonellae belonging to 39 serotypes were used (Table 1). The 65 Swedish isolates were representative of serotypes isolated from animals and feedstuffs from 1973 through 1978 (31), sewage (8), and human stools [Department of Epidemiology, National Bacteriological Laboratory, Stockholm (1957–1976)]. Upon receipt, the salmonellae were grown out on blue agar (heart infusion agar [Difco Laboratories, Detroit, Mich.] containing 1% [wt/vol] lactose and 0.003% [wt/vol] bromocresol purple) at 37°C for 18 h. Bacteria were suspended in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 15% (wt/vol) glycerol and stored at −70°C.

Cultivation of salmonellae. Strains were grown in tryptone-yeast extract (Difco Laboratories) broth medium (TY-1 medium) (18) and in a medium comprising dialysate of Trypticase soy broth (BBL Microbiology Systems), 0.05 M monosodium glutamate, and 1% (wt/vol) glycerol (23), which was deferrated by using calcium chloride as described by Bjorn et al. (3). Flasks for use with the deferrated medium (DF medium) were acid cleaned and rinsed with distilled water. Cultures were grown at 37°C for 16 to 18 h on a shaking table (100 rpm). The inocula were salmonellae grown on blue agar overnight at 37°C.

The cultures were centrifuged (3,250 × *g*, 20 min), and culture supernatant fluids were divided into two portions. One portion was stored in small volumes directly at −20°C. The other was heat treated (80°C, 30 min), cooled, and stored at −20°C as small volumes.

Enterotoxigenicity assays. (i) Cell tests. Unheated culture supernatant fluids were tested on CHO cells (15), Y1 adrenal cells (10), and Vero cells (34). Reactions were read after 16 to 18 h. All cell tests were read blind. As a check for the possibility of false-positives or subjectivity in reading, tests were performed and or read in duplicate at another Department (kindly performed by Gunnel Sigstam, Department of Bacteriology, Karolinska Institute, Stockholm). Culture supernatant fluids were used within 1 week of preparation.

(ii) Rabbit skin permeability test. Tests were performed for both the delayed permeability factor (DPF) and the rapid permeability factor (RPF) described by Sandefur and Peterson (29) by intradermal injection of 0.1-ml portions of unheated and heat-treated culture supernatant fluids, respectively. For DPF and RPF tests, rabbits were injected intravenously with 2 to 3 ml of 5% (wt/vol) Evans blue dye 16 to 18 h and 1 h post-inoculation, respectively, and the skin reactions (blueing and induration) were recorded after a further 60 and 30 min, respectively. Reactions were most readily read on the everted skin surface at necropsy. A reaction of at least 4 mm in diameter (area = 12.6 mm^2) was regarded as positive.

(iii) Infant mouse assay. Suckling mice, 3 to 4 days old, were injected intragastrically by the percutaneous route with 0.1 ml of heat-treated culture supernatant fluids containing one drop of 2% (wt/vol) Evans blue (9, 11). The ratio of the intestinal weight to the remaining body weight was determined 4 h post-inoculation. Samples yielding ratios ≥0.083 were considered as positive, ratios between 0.075 to 0.082 were considered as doubtful, and ratios ≤0.074 were considered as negative (11).

(iv) Rabbit ileal loop test. The unheated culture supernatant fluids for rabbit ileal loop tests were membrane filtered (Millipore Corp., Bedford, Mass.; 0.22 μm), and neomycin sulphate (100 μg/ml) was added to prevent bacterial contamination and growth of bacteria in loops. The procedure described by Annapurna and Sanyal (1) was followed (1 ml of filtrate per 4 to 5 cm of gut), and the fluid accumulation was read after 16 to 18 h. A volume-to-length ratio of 0.5 ml of fluid per cm of intestine was regarded as positive. Crude cholera toxin at 15 mg/ml of phosphate-buffered saline and sterile medium were included as positive and negative controls, respectively, in each rabbit.

Most CHO and Y1 adrenal cell tests were performed on at least two separate occasions. Skin permeability tests were each done on two rabbits. Strains giving positive or doubtful infant mouse tests were regrown several times, and the tests were repeated. Findings of an interesting or unusual character, e.g., positive in the CHO cell test but negative in the Y1 adrenal cell test or positive in the Y1 adrenal cell test but negative in the rabbit skin DPF test, were rechecked.

Neutralization studies. Cholera antitoxin produced in a burro (7) was kindly supplied by A.-M. Svennerholm, Department of Medical Microbiology, University of Göteborg, Göteborg, Sweden. This antitoxin contained 4,950 antitoxin units per ml.

(i) Rabbit ileal loop tests. To 1-ml aliquots of culture filtrates of salmonellae grown in DF medium 50 μl of antitoxin was added. These mixtures and controls without antitoxin were incubated at 37°C for 30 min. Mixtures and controls were inoculated into rabbit ileal loops in paired series. Fluid accumulation was read after 16 to 18 h. Neutralization was expressed as the percent reduction in fluid accumulation between the paired loops containing antitoxin-treated filtrates and controls. As a positive control, crude cholera toxin was used at 15 mg/ml of phosphate-buffered saline with and without addition of antitoxin. Negative controls contained DF medium with antitoxin.

(ii) Rabbit skin DPF tests. Mixtures of culture

TABLE 1. *List of Salmonella strains tested*

Salmonella species	No. of strains	Origin[a]	Source[b]
S. agona	3	NVI/NBL	Sewage, stool
S. anatum	2	NVI/NBL	Meat meal, stool
S. blockley	1	NVI	Sewage
S. bovis morbificans	1	NVI	Imported meat
S. braenderup	1	NVI	Sewage
S. bredeney	1	NVI	Sewage
S. california	1	NVI	Hamburger
S. cerro	1	NVI	Sewage
S. colorado	1	NVI	Sewage
S. derby	2	NVI	Mink feed, calf cadaver
S. dublin	4	NVI	Cow feces, calf
S. emek	1	CLRI	Water
S. enteritidis	4	NVI/NBL	Calf feces, stool
S. galiema	1	NVI	Sewage
S. give	1	NVI	Sewage
S. haifa (var. 1–5)	1	NVI	Sewage
S. heidelberg	1	NBL	Stool
S. indiana	1	NVI	Feather meal
S. infantis	3	NVI/NBL	Feather meal, stool
S. kaapstad	1	NVI	Sewage
S. kingston var. *copenhagen*	1	NVI	Sewage
S. kisii	1	NBL	Stool
S. liverpool	1	NVI	Unknown
S. manila	1	NVI	Food factory
S. mbandaka	1	NVI	Poultry feed
S. meleagridis	1	NVI	Duck
S. muenster	1	NVI	Sewage
S. newport	3	NVI/NBL	Imported veal, stool
S. ohio	1	NVI	Sewage
S. othmarchen	1	NVI	Sewage
S. panama	1	NVI	Sewage
S. paratyphi B	1	NBL	Stool
S. paratyphi var. *taunton*	1	NVI	Sewage
S. senftenberg	1	NVI	Feather meal
S. saint-paul	1	NVI	Sewage
S. thompson	2	NBL	Stool
S. typhimurium	12	DU/NVI/NBL	Stool, budgerigar, bullfinch
S. typhimurium var. *copenhagen*	3	NVI/NBL	Bullfinch, cow feces, stool
S. virchow	2	NBL	Stool

[a] NVI, National Veterinary Institute, Uppsala, Sweden; NBL, National Bacteriological Laboratory, Stockholm, Sweden; CLRI, Central Laboratory and Research Institute, Addis Ababa, Ethiopia; DU, Dundee University, Department of Bacteriology, Dundee, Scotland.

[b] All salmonellae from human stools were isolated from individuals either during the acute stage of diarrhea or during the convalescent stage, e.g., after returning to Sweden from a trip abroad where the individual had experienced diarrhea of up to 1 week in duration. The isolates from calves and cows were each from active cases of diarrhea, as was the *S. meleagridis* from a duck. Two cases of septicemia in bullfinches and one case of septicemia in a budgerigar yielded *Salmonella* isolates.

filtrates and antitoxin, and controls without antitoxin, were prepared in an identical manner for tests of the ability of cholera antitoxin to neutralize DPF activity in rabbit skin. Duplicate intradermal injections of mixtures and controls were performed in the same rabbit. The rabbits were injected intravenously with Evans blue dye after 16 to 18 h, and the reactions were recorded 1 h later by examination of the everted skin surface at necropsy.

(iii) CHO cell tests. Mixtures of unheated culture supernatant fluids (20 μl) and cholera antitoxin (20 μl; 100 antitoxin units) were incubated at 37°C for 30 min. These were titrated in parallel with suitably diluted control culture supernatant fluids on CHO cells, and the reactions were read after 16 to 18 h (29).

Predictor and indicator values. The method of Hall and Sebag (17) was used to calculate predictor and indicator values from four- or eightfold field tables; e.g., see Tables 2 and 4. The predictor for any test indicates the chance of a positive reaction in one test system or with one test parameter also being positive in another test system or with another test

variable. When two sets of results in systems A and B are compared,

$$\text{predictor A} = A^+ B^+/A^+ B^+ + A^+ B^-$$

$$\text{and predictor B} = B^+ A^+/B^+ A^+ + B^+ A^-.$$

The indicator value indicates the chance of negativity in two test systems or with two test parameters. When two sets of results in systems A and B are compared,

$$\text{indicator A} = A^- B^-/A^- B^- + A^- B^+$$

$$\text{and indicator B} = B^- A^-/B^- A^- + B^- A^+.$$

For example, when TY-1 medium and DF medium are compared for suitability for enterotoxigenicity tests in the CHO cell test system (see below), the predictor for TY-1 medium is calculated as $(\text{TY-1}^+ \text{DF}^+)/(\text{TY-1}^+ \text{DF}^+ + \text{TY-1}^+ \text{DF}^-)$, and the indicator is calculated as $(\text{TY-1}^- \text{DF}^-)/(\text{TY-1}^- \text{DF}^- + \text{TY-1}^- \text{DF}^+)$, where $\text{TY-1}^+ \text{DF}^+$ is the number of strains giving positive CHO cell test findings when grown in both TY-1 medium and DF medium, and so forth.

RESULTS

Medium comparison for CHO cell tests. The qualitative findings of the CHO cell test with culture supernatant fluids of salmonellae grown in DF medium and TY-1 medium revealed that the overall agreement of the tests with the two media was only 41% (28 of 68 strains), i.e., 6 positive and 22 negative findings with both media. Thirty-nine *Salmonella* strains gave positive CHO cell test findings when grown in DF medium, but negative findings when grown for TY-1 medium. One strain of *S. agona* was negative when grown in DF medium, but positive when grown in TY-1 medium. The indicator for TY-1 medium was 0.36, indicating that only 36% of the strains which were negative in the CHO cell test when grown in TY-1 medium were also negative in the CHO cell test when grown in DF medium, whereas the predictor was 86%; i.e., there was an 86% chance of a positive test with TY-1 medium also being positive with DF medium for any strain. The indicator and predictor for DF medium were 0.96 and 0.13, respectively. DF medium was subsequently chosen for comparative cell tests (66% positivity with DF medium versus 10% positivity with TY-1 medium).

CHO, Y1 adrenal, and Vero cell tests. Table 2 gives the comparative analysis of the qualitative findings in CHO and Y1 adrenal cell tests with salmonellae culture supernatant fluids from DF medium. Overall agreement was obtained for 47 of 66 strains (68%), i.e., positive or negative in both cell tests. The indicator and predictor for the Y1 adrenal cell test were 0.60 and 0.73, respectively, and for the CHO cell test, they were 0.29 and 0.91, respectively.

TABLE 2. *Comparative qualitative results in the CHO cell and Y1 adrenal cell test systems with culture supernatant fluids of Salmonella strains grown in DF medium*

Y1 adrenal cell test result	No. of strains with following result by CHO cell test	
	Positive	Negative[a]
Positive	41	15[b]
Negative	4[c]	6[d]

[a] Excludes one strain each of *S. dublin* and *S. paratyphi* var. *taunton* which were not tested on Y1 adrenal cells after growth in DF medium.

[b] Includes *S. agona*, which was negative on CHO cells when grown in DF medium but positive in TY-1 medium. Strains were *S. agona* (2), *S. anatum*, *S. enteritidis* (2), *S. heidelberg*, *S. liverpool*, *S. newport*, *S. typhimurium* (5), and *S. typhimurium* var. *copenhagen* (2).

[c] *S. anatum*, *S. bovis morbificans*, *S. newport*, and *S. typhimurium*.

[d] *S. blockley*, *S. braenderup*, *S. bredeney*, *S. newport*, *S. ohio*, and *S. panama*.

Comparative data in the three cell tests for enterotoxigenicity of salmonellae revealed that 85% (56 of 66 strains tested) were positive in the Y1 adrenal cell test, 66% were positive (45 of 68 strains tested) in the CHO cell test, and 69% were positive (31 of 45 strains tested) in the Vero cell test. In the Y1 adrenal cell test, the majority of positive reactions were seen up to a 1:10 dilution of culture supernatant fluids. Eighteen strains gave positive reactions at a 1:100 dilution on at least one occasion. Titers of culture supernatant fluids on CHO and Vero cells were, in general, similar when tested in parallel with Y1 adrenal cells, although the latter yielded higher titers in some instances. In many cases, undiluted culture supernatant fluids yielded negative findings or were cytotoxic for the tissue culture cells, whereas 1:10 dilutions of supernatant fluids gave unequivocal positive findings.

Cell tests compared with the DPF rabbit skin test. When the Y1 adrenal cell test findings were compared with the DPF skin test results with culture supernatant fluids from DF medium (Table 3), the overall agreement was 73% (48 of 66 strains). The indicator and predictor for DPF activity compared with Y1 adrenal cell test reactivity were 0.28 and 0.90, respectively, and for the Y1 adrenal cell test compared with the DPF skin test, they were 0.50 and 0.77, respectively. Of the salmonellae tested, 73% produced DPF.

When the CHO cell test results were compared with the rabbit skin DPF activity, the overall agreement was 71% (47 of 66 strains). The indicator and predictor for DPF activity

TABLE 3. *Comparison of qualitative findings in Y1 adrenal cell, CHO cell, and rabbit skin DPF tests with culture supernatant fluids of Salmonella strains grown in DF medium*[a]

Rabbit skin DPF test result	No. of strains giving the indicated cell test profile			
	$Y1^+$ CHO^+	$Y1^+$ CHO^-	$Y1^-$ CHO^+	$Y1^-$ CHO^-
Positive	33	10	4[b]	1[c]
Negative	8[d]	5[e]	0	5

[a] Not included were one strain which was not tested on Y1 adrenal, but was CHO^+ DPF^-, *S. dublin*, and one strain which was not tested in both Y1 adrenal and DPF tests, but was CHO^-, *S. paratyphi* var. *taunton*.

[b] See footnote *c* in Table 2.

[c] *S. blockley*; cf. footnote *d* in Table 2.

[d] *S. dublin* (2), *S. emek*, *S. enteritidis*, *S. galiema*, *S. give*, *S. typhimurium*, and *S. typhimurium* var. *copenhagen*.

[e] *S. anatum*, *S. heidelberg*, *S. liverpool*, *S. typhimurium*, and *S. typhimurium* var. *copenhagen*; cf. footnote *b* in Table 2.

TABLE 4. *Comparison of rabbit ileal loop reactivity with reactivity in Y1 adrenal and DPF skin tests of Salmonella culture supernatant fluids from DF medium*

Rabbit ileal loop test result	No. of strains giving indicated Y1 adrenal/DPF test result			
	$Y1^+$ DPF^+	$Y1^+$ DPF^-	$Y1^-$ DPF^+	$Y1^-$ DPF^-
Positive	25[a]	4[b]	2[c]	1[d]
Negative	2[e]	0	0	1[f]

[a] Of these, 14 produced RPF (also see Table 7), namely, *S. agona*, *S. california*, *S. enteritidis*, *S. haifa*, *S. indiana*, *S. infantis*, *S. kisii*, *S. muenster*, *S. paratyphi* B, *S. saint-paul*, *S. thompson*, and *S. typhimurium* (3).

[b] *S. emek*, *S. enteritidis*, and *S. typhimurium*; also included was one strain of *S. dublin* which was not tested in the Y1 adrenal cell test but was positive in the CHO cell test.

[c] *S. blockley* and *S. bovis morbificans*.

[d] *S. ohio*.

[e] *S. derby* (2).

[f] *S. newport*.

were 0.56 and 0.77, respectively, and for the CHO cell test, they were 0.48 and 0.82, respectively.

The overall agreement of findings in these two cell tests and the rabbit skin DPF test, i.e., CHO^+ $Y1^+$ DPF^+ and CHO^- $Y1^-$ DPF^-, was 58% (38 of 66 strains). DPF blueing activity ranged from 20 to 531 mm^2, and the induration activity ranged between 20 to 345 mm^2 (Fig. 1).

Rabbit ileal loop tests compared with Y1 adrenal and DPF tests. The results of comparative tests in the Y1 adrenal, rabbit skin DPF, and rabbit ileal loop test systems are shown in Table 4. Only a limited selection of strains were run in the ileal loops, i.e., 34 of 66 strains tested in both the Y1 adrenal and rabbit skin DPF tests. The indicator and predictor for rabbit loop tests compared with DPF tests were 0.33 and 0.84, respectively, and for DPF tests compared with rabbit loop tests, they were 0.17 and 0.93, respectively. When rabbit ileal loop test findings were compared with results in the Y1 adrenal cell test, the indicators and predictors were 0.33 and 0.94, respectively, for both tests. The indicators, however, may be unreliable because fewer negative strains in either or both of the Y1 adrenal and DPF tests were selected for rabbit ileal loop testing.

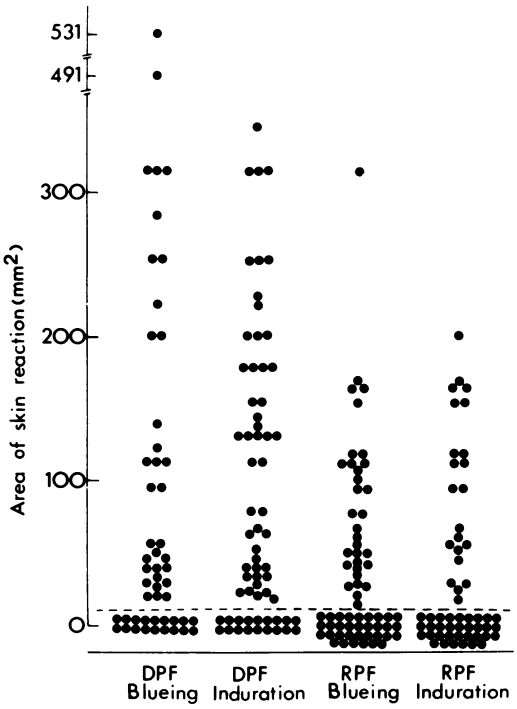

FIG. 1. *Distribution of blueing and induration reactions in rabbit skin permeability tests for DPF and RPF with culture supernatant fluids of different salmonellae. The broken line denotes the limit for positivity in the tests.*

Infant mouse and rabbit skin RPF tests. Both of these tests were performed with heat-treated culture supernatant fluids. The RPF tests were done with both TY-1 medium and DF medium for 50 strains. The overall agreement between media was 54%, and with either medium, about 50% of the salmonellae were positive for RPF. Including an additional 18 strains tested only in TY-1 medium or DF medium, 66% of the *Salmonella* strains were positive for RPF,

based on positivity when grown in one or both of these media.

The infant mouse test and the RPF test were employed simultaneously for screening heat-treated samples of culture supernatant fluids from TY-1 medium. The overall agreement was 54% (Table 5). Comparing the infant mouse and RPF tests, the indicator and predictor for RPF were 0.93 and 0.09, respectively, and for infant mouse test reactivity, they were 0.54 and 0.50, respectively.

RPF blueing activity ranged between 15 to 167 mm^2, and induration ranged from 19 to 201 mm^2 (Fig. 1).

DPF and RPF rabbit skin activities. A fourfold field comparison (Table 6) showed that of 65 strains, 35% were positive in both the DPF and RPF skin tests. Of the DPF-positive strains, approximately 50% were also positive in the RPF test. Of the RPF-positive strains, 74% were also positive for DPF activity. The indicators for DPF and RPF were 0.50 and 0.27, respectively, and for DPF and RPF, they were 0.49 and 0.72, respectively.

TABLE 5. *Comparison of qualitative findings in infant mouse test and rabbit skin RPF test with Salmonella culture supernatant fluids from TY-1 medium heat treated at 80°C for 30 min*

Rabbit skin RPF test result	No. of strains with indicated reaction in infant mouse test	
	Positive	Negative
Positive	2[a]	21[b]
Negative	2[c]	25

[a] *S. dublin* and *S. saint-paul.*

[b] *S. agona, S. anatum, S. braenderup, S. bredeney, S. california, S. colorado, S. derby, S. emek, S. enteritidis, S. indiana, S. infantis* (2), *S. liverpool, S. meleagridis, S. ohio, S. othmarchen, S. typhimurium* (4), and *S. typhimurium* var. *copenhagen.*

[c] *S. heidelberg* and *S. mbandaka.*

TABLE 6. *Comparison of production of DPF and RPF rabbit skin activities by Salmonella strains*

DPF rabbit skin activity	No. of strains with indicated RPF activity	
	Positive	Negative
Positive	23	24[a]
Negative	9[b]	9[c]

[a] *S. agona* (2), *S. blockley, S. bovis morbificans, S. cerro, S. derby, S. dublin, S. enteritidis* (2), *S. kaapstad, S. kingston* var. *copenhagen, S. manila, S. mbandaka, S. newport, S. thompson, S. typhimurium* (5), *S. senftenberg, S. typhimurium* var. *copenhagen,* and *S. virchow* (2).

[b] *S. braenderup, S. bredeney, S. dublin* (2), *S. emek, S. liverpool, S. ohio, S. typhimurium,* and *S. typhimurium* var. *copenhagen.*

[c] *S. dublin, S. enteritidis, S. galiema, S. give, S. heidelberg, S. newport, S. panama, S. typhimurium,* and *S. typhimurium* var. *copenhagen.*

Neutralization tests. Moderate to strong neutralization of rabbit ileal loop activity of culture filtrates was obtained with anticholera antitoxin with seven of the nine strains tested. The two strains which were comparatively poorly neutralized were positive in the Y1 adrenal, CHO, and DPF tests (*S. dublin* and *S. infantis*).

Culture filtrates of 11 DPF-positive *Salmonella* serotypes (12 strains) were completely neutralized in DPF tests in rabbit skin by cholera antitoxin. The CHO cell reactivity of culture supernatant fluids of these same 12 strains were also abolished by cholera antitoxin.

Enterotoxigenicity patterns of selected *Salmonella* isolates. A summary of the results for the *Salmonella* species represented by more than one strain is presented in Table 7. The most notable feature is that 31 of these isolates were positive for DPF. Of the *S. typhimurium,* including strains designated var. *copenhagen,* 11 of 15 were positive for DPF and 7 were positive for RPF, only two of the latter being negative for DPF.

Enterotoxigenicity of salmonellae in terms of source. Of the 28 *Salmonella* isolates from human stools, 82% were positive for DPF, 92% were positive in the Y1 adrenal cell test, and all of 11 tested were positive in the rabbit ileal loop test. Three of 3 tested *S. enteritidis* strains and 7 of 10 *S. typhimurium* isolates were positive in both Y1 adrenal and DPF tests. Eleven stool isolates, including 10 which were DPF positive, produced RPF.

Of 17 sewage isolates, 63% were positive for DPF, and 60% were positive in the Y1 adrenal cell test. Eight of eight tested were positive in the rabbit ileal loop test. All 17 isolates were of different serotypes and did not include *S. typhimurium* or *S. enteritidis.* Six of nine RPF-positive strains also produced DPF. These findings indicate that enterotoxigenicity is relatively common among salmonellae which are rarely isolated from human stools in Sweden (8).

DISCUSSION

Enterotoxigenicity, as detected by three cell tests, two rabbit skin tests, the infant mouse test, and the rabbit ileal loop test, appears to occur commonly among Swedish *Salmonella* isolates. Only two of the 68 salmonellae examined, namely, *S. newport* and *S. panama,* were negative in all tests.

The rabbit skin DPF test has been proposed as a rapid screening assay for enterotoxigenicity

TABLE 7. *Summary of the Y1 adrenal and CHO cell test findings, DPF and RPF activities, infant mouse assays, and rabbit ileal loop tests for 40 strains representing multiple isolates of individual species*[a]

Salmonella species	No. of strains	Cell test		Rabbit skin reactivity		Infant mouse test	Rabbit ileal loop test
		Y1	CHO	DPF	RPF		
S. agona	2	+	−	+	−	−/NT	NT
	1	+	+	+	+	−	+
S. anatum	1	−	+	+	+	−	NT
	1	+	−	−	NT	NT	NT
S. derby	2	+	+	+	−/+	−	−
S. dublin	1	+	+	−	−	+	+
	1	NT	+	−	+	NT	+
	2	+	+	−	+/−	+/NT	NT
S. enteritidis	2	+	+	+/−	+/−	−	+
	2	+	−	+	−	−	NT
S. infantis	3	+	+	+	+[2]/NT	−[2]/NT	+/NT[2]
S. newport	1	−	−	−	−	−	−
	1	−	+	+	+	NT	NT
	1	+	−	+	−	−	NT
S. thompson	2	+	+	+	+/−	NT	+
S. typhimurium	4	+	+	+	+	−[3]/NT	+[3]/NT
	1	+	+	+	−	−	+
	1	+	+	−	+	−	+
	3	+	−	+	−	−/NT[2]	NT
	1	+	−	+	+	NT	NT
	1	−	+	+	−	−	NT
	1	+	−	−	−	NT	NT
S. typhimurium var. *copenhagen*	2	+	−	+/−	−	−	+/NT
	1	+	+	−	+	−	NT
S. virchow	2	+	+	+	−	−	+

[a] NT, Not tested. Test findings given before and after the shill refer to the respective strains across the table; superior numbers indicate the number of strains giving the indicated reaction in each test.

among salmonellae (22). Kühn et al. (22) demonstrated DPF production in 205 of 378 *Salmonella* isolates representing 21 serotypes. Herein, 48 of 67 tested isolates, representing 28 serotypes, were DPF positive. With the exception of *S. paratyphi* B, enterotoxigenic isolates were found among nine serotypes common to both investigations.

Sandefur and Peterson (30) reported that a partially purified preparation of DPF caused elongation of CHO cells and that both activities were neutralized by antiserum to cholera toxin. However, they were unable to demonstrate the CHO cell elongation factor in crude culture filtrates. An inhibitory substance was apparently removed by gel filtration chromatography of such filtrates. Using crude culture filtrates of *S. typhimurium, S. enteritidis agona, S. newport,* and *S. paratyphi,* Donta and Smith (10) failed to show morphological changes in Y1 adrenal cells or induction of steroidogenesis. In contrast, in the present investigation, morphological changes in Y1 adrenal, CHO, and Vero cells, typical of those induced by *E. coli* heat-labile enterotoxin and by cholera toxin, were demonstrated directly with culture supernatant fluids of salmonellae. Since the culture supernatants gave rise to positive cell tests in dilutions of 1:10 and even 1:100, the effects of inhibitory substances may have been obviated by dilution.

Sedlock et al. (33) observed that, although *Salmonella* isolates grew well in eight different complex and two different synthetic media, enterotoxin production as measured by the rabbit

ileal loop assay was not detected in culture filtrates of each medium. In the present study, a deferrated medium (3, 4), containing about 0.05 μg of iron per ml, yielded a sevenfold higher number of positives in CHO cell tests than did TY-1 medium (0.6 μg of iron per ml), which has been previously shown to be a good medium for production of *E. coli* heat-labile enterotoxin (41). Concentrations of iron similar to that in TY-1 medium have been shown to decrease yields of extracellular exotoxin A, proteases, and hemagglutinin produced by *P. aeruginosa* (3, 4). Bjorn et al. (3, 4) suggested that iron regulates release of the latter pseudomonad metabolites by an iron-sensitive mechanism. Moreover, addition of iron to growth media has also been shown to depress production of the lecithinase (α-toxin) of *Clostridium perfringens,* the neurotoxin of *Shigella shigae,* diphtheria toxin, and tetanus toxin (6). Furthermore, *E. coli* heat-labile enterotoxin has been shown to be cell wall associated or periplasmically located, and its detection depends, accordingly, on its release (42). Thus, supplementation of media with iron (33) may adversely affect synthesis or release or both of *Salmonella* enterotoxin.

Koupal and Deibel (21) and Kühn et al. (22) have described an enterotoxic factor in *S. enteritidis* and *S. typhimurium,* respectively, that caused intestinal fluid accumulation in the infant mouse model. The factor appeared to be heat-stable. In contrast, Peterson and co-workers have been unable to detect such a factor (24a; unpublished data). However, in the current investigation, four isolates, one each of *S. dublin, S. heidelberg, S. mbandaka,* and *S. saint-paul,* were shown to produce a heat-stable factor giving a positive response in infant mice. Thus, it appears that this model gives disparate findings. Indeed, although initially reported to yield reproducible results with culture filtrates of *Salmonella* isolates in contrast to other animal models (21), the infant mouse assay, as reported by Sedlock and Deibel (32), often exhibits erratic responses. Moreover, like *E. coli* heat-stable enterotoxins, which have been shown to vary in their reactivities in different animal models (5, 16, 20, 24), heat-stable enterotoxins elaborated by salmonellae may show analogous variation.

Twenty-four *Salmonella* serotypes produced RPF. Seven of these serotypes were represented among the RPF-positive species found by Sandefur and Peterson (29). Of 32 RPF-positive strains, 3, namely, 1 each of *S. ohio, S. braenderup,* and *S. bredeney,* were negative in the cell tests, in the infant mouse test, and for DPF; 1 of these 3 was positive in the rabbit ileal loop test. The latter findings suggest that RPF-only (heat-stable enterotoxin-only) strains of salmonellae are rare and that RPF is enterotoxic. Indeed, Takeda et al. (35) reported a rapid skin permeability test in guinea pigs for demonstration of *E. coli* heat-stable enterotoxin, which is known to be enterotoxic in mice and rabbits (5, 9, 11, 16).

Culture filtrates of all but two (both *S. derby*) of the tested strains giving positive reactions in the Y1 adrenal and/or DPF skin test(s) caused fluid accumulations in rabbit ileal loops (Table 4). Of these 33 isolates, 18 also produced RPF. Moreover, CHO cell reactivity, DPF activity in rabbit skin, and rabbit ileal loop reactivity of unheated culture filtrates were each neutralized by anticholera antitoxin. It would appear that the factor causing morphological changes in Y1 adrenal or CHO cells or both and responsible for DPF activity (30) can also cause fluid accumulation in intestinal loops. This has not been made clear by the studies on partially purified preparations of DPF from *S. typhimurium* (25, 29, 39), by the screening survey of Kühn et al. (22), or by the studies on *S. enteritidis* enterotoxin (21, 32, 33). Moreover, *S. blockley* was negative in all three cell tests, in the infant mouse test, and for RPF; yet it was reproducibly positive for DPF and in rabbit ileal loops. Washing the intestinal lumen of rabbits with a mucolytic agent before administration of toxin has been reported to enhance the fluid accumulation response (32). Satisfactory ileal loop responses in rabbits, however, were obtained in the present study without such washing.

The Y1 adrenal cell test appears from this work to be more sensitive than the CHO and Vero cell systems. However, with respect to predictor values for comparisons of Y1 adrenal, DPF, and rabbit loop activities, a positive finding in the DPF test is more likely to be positive in both of the others than vice versa. Thus, on the basis of these findings, rabbit skin inoculations for both DPF and RPF provide the most reliable means of screening for both factors, even though 17 to 28% of DPF-negative strains may be positive in either the Y1 adrenal or rabbit ileal loop test (some rabbit loop tests may be positive because of RPF activity).

The investigations of Giannella and colleagues (12–14, 27) on *S. typhimurium* have led to the following conclusions: (i) stimulation of adenylate cyclase causes ileal secretion; (ii) invasion of the intestinal mucosa does not in itself activate adenylate cyclase; (iii) plasma filtration through a damaged mucosa does not contribute to the *Salmonella*-induced intraluminal fluid accumulation; (iv) the acute mucosal inflammatory reaction induced by *Salmonella* isolates appears

to play a role in ileal secretion; and (v) prostaglandins released by the inflammatory process may induce intestinal secretion. The apparently widespread distribution of enterotoxigenicity among salmonellae, as evidenced in the present investigation and by others (22, 29, 32), strongly suggests the possibility of direct stimulation of adenylate cyclase without excluding activation of this enzyme by other mediators, such as prostaglandin(s).

Indeed, the inhibition of DPF-induced elongation of CHO cells by anticholera antitoxin (30), the inhibition of DPF activity by both anticholera antitoxin and purified ganglioside preparations containing G_{M1} ganglioside (25), and the inhibition of gut loop activity, CHO cell reactivity, and DPF activity in the present study by anticholera antitoxin all point to a cholera toxin-like activation of adenylate cyclase in intestinal epithelial cells. Furthermore, the possibility that enterotoxin is located in the cell wall or outer membrane fraction of *Salmonella* bacteria (21) may explain why adenylate cyclase activation occurs concomitantly with invasion (13, 14). In addition, the typical morphological responses in Y1 adrenal, CHO, and Vero cells induced by exposure to culture supernatant fluids of salmonellae provide further evidence of the production of a factor, possibly DPF, capable of direct adenylate cyclase activation. Although Sandefur and Peterson (25) failed to demonstrate fluid accumulation in rabbit intestinal loops injected with DPF, the neutralization of filtrates by anticholera antitoxin herein supports the view that DPF is enterotoxic. The roles of DPF, RPF, and other factors in the fluid secretory phase of *Salmonella* infection awaits clarification.

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