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Biological Evaluation of a Methanol-Soluble, Heat-Stable *Escherichia coli* Enterotoxin in Infant Mice, Pigs, Rabbits, and Calves

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Escherichia coli P16 was shown to produce two heat-stable toxins (ST) with differing biological activity. The toxins were separated by methanol extraction, and the first, STa, was methanol soluble, partially heat stable, active in neonatal piglets (1 to 3 days old) and infant mice, but inactive in weaned pigs (7 to 9 weeks old); the second, STb, was methanol insoluble, active in weaned pigs and rabbit ligated loops, but inactive in infant mice. It is therefore suggested that use of suckling mice as indicators of ST production will fail to identify certain ST-producing strains.

Strains of *Escherichia coli* enteropathogenic for pigs have been shown to secrete enterotoxins in vitro (15, 21, 32, 35). These enterotoxins cause fluid accumulation when introduced into ligated intestinal loops prepared in rabbits or pigs (7, 15, 21, 32, 33).

The strains of *E. coli* involved are believed to produce two different types of enterotoxin: a heat-labile, non-dialyzable, immunogenic toxin (LT) and a heat-stable, dialyzable toxin (ST) (2, 7, 11, 13, 32, 35). In vitro all enteropathogenic strains of *E. coli* isolated from pigs produce ST, and some also produce LT (7, 32).

We have been interested in purifying the ST secreted by porcine strains of enteropathogenic *E. coli* and have used the infant mouse assay for its detection (3, 4, 8, 13).

However, during our initial purification of the ST toxin, we found anomalies which suggested the presence of more than one toxin whose effects varied with the age of piglets challenged. This paper details the results of our investigations of this finding and also includes some preliminary experiments performed with a strain of *E. coli* enteropathogenic for calves.

MATERIALS AND METHODS

Organisms. *E. coli* strains P16 (serotype O9:K9), P307 (O8:K87:K88a,b), Abbotstown (O149:K91:K88a,c), B44 (O9:K90:K99), 431 (O101:K?), 637 (O64:K?), 613 (O101:K?), P2 (untypable), F11 (O18:K?: H14), P155 (O149:K91:K88a,c:H10), and F11/P155 (the F11 organism with the Ent$^+$ plasmid from P155) were obtained from H. W. Smith (Houghton Poultry Research Station, Houghton, Huntingdonshire, England).

Media. The defined medium of Mitchell et al. (19) containing 0.5% glucose was used in the large-scale preparation of enterotoxin material from *E. coli* strain P16 and for the preparation of enterotoxin from *E. coli* strain B44. All other fermentations were performed with brain heart infusion broth (Oxoid).

Enterotoxin production. Enterotoxin from *E. coli* strain P16 was produced as previously reported (22). All other fermentations were performed in 100-ml volumes with 500-ml conical flasks as follows: 10 ml of peptone-water was inoculated with the *E. coli* strain from a Dorset egg slope and incubated for 18 h at 37°C. A 1% inoculum of this overnight culture was added to the sterile medium and incubated for 24 h at 37°C in a shaking incubator (200 rpm).

After centrifugation (23,000 × *g*) for 30 min at 4°C, the clarified, decanted supernatants were heated at 65°C for 15 min, concentrated 10-fold by rotary evaporation, and sterile filtered. The sterility of all final products was checked.

Methanol extraction of ST toxin. The methanol extraction of ST was performed as detailed by Mullan et al. (22). The methanol-insoluble material was reconstituted to the volume of the extracted culture filtrate. In all animal models this material was tested at an equivalent volume (and osmolarity) to the original culture filtrate (i.e., equivalent concentration).

The sterility of all products was checked, and the absence of residual methanol in the samples was confirmed by gas-liquid chromatography.

Assays for enterotoxigenic activity. (i) Suckling mouse assay. The suckling mouse assay was performed essentially as detailed by Dean et al. (3) with the modifications previously reported (22). Essentially, 0.05 ml of sample was dosed to groups of infant mice, and the animals were killed 2 h later and ratios of gut weight to body weight were determined.

(ii) Weaned pig ligated loop assay. The technique used for the weaned pig ligated loop assay was basically that of Smith and Halls (31). Pigs 7 to 9 weeks old were used, the length of the loops was approximately 10 cm, and 6 ml of test sample was used in each loop. The activity of the sample is expressed

as the volume/length ratio 18 h later at postmortem. A maximum of 18 loops was used in each pig, and samples of equivalent potency (mouse units [MU]) were of similar osmolarity.

(iii) Neonatal pig ligated loop assay. The technique used for the neonatal pig ligated loop assay was similar to the technique used for the weaned pig. Pigs 1 to 3 days old were used, and an inoculum of 2.5 ml of test sample was injected into each loop. A maximum of 12 loops were used in each pig. Again, all of the samples were iso-osmotic (340 mosmol/liter).

(iv) Piglet oral dosing assay. The procedure followed for the piglet oral dosing assay was that of Kohler (15). Colostrum-fed piglets aged 1 to 3 days were dosed intragastrically with 25 ml of sample adjusted to an osmolarity of 130 to 140 mosmol/liter. Only litters passing normal feces were used. After dosing, the piglets were allowed to suckle ad lib and were observed for diarrhea at 30-min intervals for 7 h. Animals were recorded as diarrheic if they produced fluid feces for at least 1 h.

(v) Rabbit ligated loop assay. The method used for the rabbit ligated loop assay was that of Evans et al. (7) using a 5-h assay time. Four adult rabbits were used, and 12 ligated loops were prepared in each animal. The inoculum volume was 2 ml per loop, and the osmolarities of the solutions were 800 to 850 mosmol/liter.

(vi) Calf ligated loop assay. The method used for the calf ligated loop assay was that of Smith and Halls (31). Two calves were taken at 1 to 2 weeks of age, 24 loops were prepared in each animal, the inoculum was 3 ml per loop, and each sample was tested adjacent to its respective control. The osmolarity of the solutions was 370 to 380 mosmol/liter. The animals were killed and postmortemed 18 h later.

Statistical tests. The tests used were analysis of variance, independent *t* test, and the 2 × 2 contingency test.

RESULTS

Infant mouse assay. The activities of toxin samples are shown in Table 1. Toxins from strains P307 and Abbotstown were consistently negative when assayed in the infant mouse model, all other strains tested for toxin production (except the non-enterotoxigenic F11) were positive, and the activity was methanol soluble.

Dose responses on the P16 toxin and its methanol extract have been previously reported (22). We have defined a 50% response (ratio of gut weight to body weight of 0.105 in infant mice) as 1 unit of toxin activity.

Weaned pig ligated loop assay. It was of interest to determine whether the methanol extract of P16 ST was also active in pigs. Initially the effect was studied in ligated loops of 8- to 9-week-old pigs. The results are shown in Fig. 1. The toxin methanol precipitate, inactive in baby

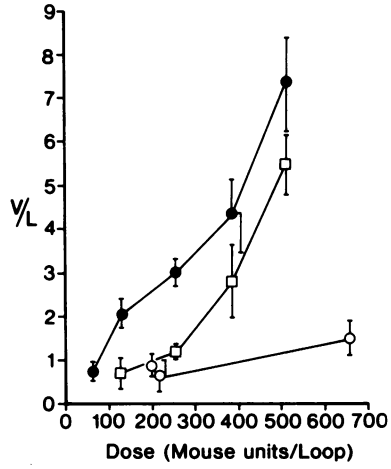

FIG. 1. *Enterotoxigenic activity of the crude P16 culture filtrate, and its methanol fractions in weaned pig ligated loops. Symbols: ●, culture filtrate; □, methanol precipitate; ○, methanol extract. Each point represents the mean ± standard error of the mean of the ratio of volume to length of at least six loops. The methanol-precipitated material, inactive in mice, was dosed at an equivalent concentration to the culture filtrate.*

TABLE 1. *Enterotoxigenic activity of culture filtrates and their extracts in the baby mouse model*

Strain	GW/BW[a]		
	Culture filtrate	Methanol extract	Methanol precipitate
P16	0.131 ± 0.010	0.113 ± 0.004	0.057 ± 0.001
B44	0.128 ± 0.008	0.116 ± 0.005	0.061 ± 0.001
Abb	0.050 ± 0.001	0.051 ± 0.002	0.060 ± 0.004
P307	0.053 ± 0.001	0.048 ± 0.001	0.056 ± 0.002
F11	0.057 ± 0.005	0.056 ± 0.001	0.052 ± 0.001
F11/P155	0.131 ± 0.006	0.141 ± 0.012	0.061 ± 0.002
431	0.123 ± 0.010	0.141 ± 0.004	0.052 ± 0.001
637	0.119 ± 0.009	0.115 ± 0.005	0.052 ± 0.002
613	0.121 ± 0.006	0.139 ± 0.005	0.054 ± 0.003
P2	0.137 ± 0.005	0.128 ± 0.005	0.055 ± 0.004
P155	0.135 ± 0.003	0.132 ± 0.004	0.062 ± 0.003

[a] GW/BW, Ratio of gut weight to body weight. The mean ± standard error of the mean of five mice dosed with 0.05 ml of sample and postmortemed 2 h later.

mice, was tested at an equivalent concentration to the unextracted toxin and is shown here for comparison. It may be seen that in this model the methanol-soluble material was inactive and that most of the activity was retained in the methanol-precipitated material. This suggested that the crude toxin material contained two types of ST. The mouse-inactive culture filtrate from strain P307 was tested, and the result is shown in Table 2, along with the result obtained with the P16 culture filtrate dosed at equal volumes (2 ml of crude filtrate diluted to 6 ml before test). Here the P307 ST (mouse inactive) showed similar dilating ability to the P16 ST (mouse active). The methanol-soluble ST was further evaluated in baby piglets.

Neonatal piglet ligated loop. The results obtained in the neonatal piglet ligated loop are shown in Table 3. The methanol extract was of comparable activity to the crude toxin in this model, the samples tested contained equivalent MU per milliliter (approximately 30 MU/ml). By analysis of variance, the crude toxin and its methanol extract were not significantly different but were both significantly active over their respective controls ($P < 0.001$).

Oral dosing of neonatal piglets. As in the young pig ligated loop assay above, the toxin and its methanol extract were dosed at equivalent MU per pig (approximately 300 MU/pig). The methanol precipitate material was dosed at an equivalent concentration to the unextracted toxin. The results are shown in Table 4.

As in the neonatal piglet ligated loop assay, the active material was methanol soluble. A 2 × 2 contingency test showed that the unextracted toxin was significantly more active than its control, or the other controls, and the methanol-precipitated material (all $P < 0.05$). However, the unextracted material was not significantly different from the methanol-soluble material.

Rabbit ligated loop assay. The results obtained in the rabbit ligated loop are shown in Table 5. The methanol precipitate was of comparable activity to the culture filtrate in this model, whereas the methanol extract showed little activity. The samples contained equivalent MU per milliliter (approximately 120 MU per loop). The toxin and its methanol precipitate were not significantly different (analysis of variance), but both were significantly more active than either the methanol-soluble toxin or uninoculated control medium ($P < 0.001$).

Calf ligated loop assay. In light of the results with P16, it was of interest to evaluate the activity of the methanol-soluble calf ST (from *E. coli* strain B44) in the calf ligated loop assay. The results are shown in Table 6.

It can be seen that the methanol-soluble toxin was active, whereas the methanol-insoluble toxin was inactive. This agrees with the results obtained from the infant mouse assay.

TABLE 2. *Enterotoxigenic activity of crude P307 and P16 culture filtrates in weaned pig ligated loops*

Strain	V/L[a]
P16[b]	2.08 ± 0.37 (36)
P307[b]	3.28 ± 0.61 (17)
Control[c] medium	0.51 ± 0.14 (12)

[a] V/L, Ratio of volume to length ± standard error of the mean. Figures in parenthesis indicate number of loops inoculated.

[b] The osmolarity of each sample was 340 mosmol/liter.

[c] Uninoculated control medium at same concentration as the culture filtrates.

TABLE 3. *Enterotoxigenic activity of the crude P16 culture filtrate and its methanol extract in neonatal pig ligated loops*

Sample[a]	V/L[b]
Crude culture filtrate	1.16 ± 0.18 (15)
Crude culture filtrate control[c]	0.21 ± 0.05 (15)
Methanol extract	1.07 ± 0.18 (12)
Methanol extract control[d]	0.14 ± 0.07 (12)

[a] The osmolarity of each sample was 340 mosmol/liter.

[b] V/L, Ratio of volume to length ± standard error of the mean. Figures in parentheses indicate number of loops inoculated.

[c] Uninoculated culture medium at same concentration as the toxin culture filtrate.

[d] Methanol-extracted, uninoculated culture medium.

TABLE 4. *Enterotoxigenic activity of P16 culture filtrate and methanol fractions when given orally to neonatal piglets*

Sample	No. of animals diarrheic/total no. dosed[a]	
	Inoculated medium	Uninoculated medium
Crude filtrate	4/4	(0/4)
Methanol extract	5/5	(0/4)
Methanol precipitate	0/4	(0/4)

[a] Animals were considered positive if diarrheic for longer than 1 h.

DISCUSSION

The infant mouse assay is widely used as a screen for ST-producing enteropathogenic *E. coli*. The use of this model has, however, given rise to certain results which conflict with data

TABLE 5. *Enterotoxigenic activity of the crude P16 culture filtrate and its methanol fractions in the rabbit ligated loop*

No.	Sample	V/L[a]	Sample no. of comparison	P[b]
1	Culture filtrate	1.37 ± 0.08	2	NS[c]
			3	<0.001
			4	<0.001
2	Methanol precipitate	1.15 ± 0.11	3	<0.001
			4	<0.001
3	Methanol extract	0.74 ± 0.06	4	NS
4	Uninoculated control medium	0.63 ± 0.06		

[a] Mean ± standard error of the mean of ratio of volume to length (V/L) from eight loops.
[b] Analysis of variance.
[c] NS, Not significant.

TABLE 6. *Enterotoxigenic activity of B44 culture filtrate and its fractions in the calf ligated loop model*

No.	Sample	V/L[a]	Sample no. of comparison	P[b]
1	Culture filtrate	7.8 ± 0.4	2	<0.001
			3	<0.01
			5	<0.001
2	Control medium	2.3 ± 0.4		
3	Methanol-soluble culture filtrate	5.8 ± 0.6	4	<0.001
			5	<0.005
4	Methanol-soluble control medium	1.2 ± 0.3		
5	Methanol-insoluble culture filtrate	2.7 ± 0.5	6	NS[c]
6	Methanol-insoluble control medium	2.2 ± 0.4		

[a] Mean ± standard error of the mean of ratio of volume to length (V/L) from eight loops.
[b] Independent *t* test.
[c] NS, Not significant.

collected from other animal models. When the heat stability of ST was tested in the earlier animal models (6- to 9-week-old pig or rabbit ligated loop), it was stable to 100°C for 30 min (1, 6, 7, 14, 23, 24, 27, 32) and showed some stability even at 121°C (24, 32). However, when the heat stability of ST was assayed in the infant mouse model, the results were less clear cut. Some workers using this technique observed no decrease in activity after 15 min at 100°C (5, 10), whereas others observed either a reduction in activity (8, 15, 22) or even a complete loss of the activity (13, 35). The variation of heat stability of toxin activity in rabbit ligated loops and mice has been suggested as arising from two distinct STs (10). The possibility of two forms of ST is supported by Moon and Whipp's classification of enteropathogenic *E. coli* into two distinct classes (20). This suggested that strains which produced toxin active in pigs of all ages should be classified as class I enteropathogens, whereas those producing toxin active only in baby piglets should be classified as class II enteropathogens. This distinction has also been reported by other workers (12, 33, 34), and some biochemical differences have been found between class I and II enteropathogens (17). We have shown that the atypical strains of Smith and Linggood (33) produce a toxin which is active in infant mice and is methanol soluble. Smith and Linggood (33) have shown that their class II enteropathogens, strains 431, 613, 637, and P2, produce an ST which has a similar activity to ST produced by calf and lamb strains of *E. coli*.

Sivaswamy and Gyles (30) have shown that the ability of the mouse test to detect ST parallels that of the calf ligated loop and found 27 strains capable of secreting an ST active in pigs but inactive when assayed in the baby mouse model. The use of the baby mouse model for the detection of ST has also resulted in strains of *E. coli* being reported as producing only LT (10, 26, 28), but workers using other detection assays for ST have failed to find any *E. coli* strains producing only LT (16, 23, 27). In our own hands, strain P307 was repeatedly negative when assayed for ST production by using the infant mouse assay, thus confirming the work of Jacks and Wu (13). However, we have found toxin which, although inactive in baby mice, was active in 6- to 9-week-old pigs. Indeed, from mouse and rabbit results alone, P307 would be designated (incorrectly) as producing only LT, whereas results from pigs would show it as producing both ST and LT.

We therefore believe that enterotoxigenic *E. coli* can secrete two forms of ST: STa, which is partially heat stable, active in infant mice and neonatal piglets, and methanol soluble; and STb, which is heat stable, inactive in baby mice, methanol insoluble, and active in ligated intestines of older piglets and rabbit ligated loops. Our results may then be interpreted as follows. *E. coli* strain P16 secretes STa and STb, and on extraction the mouse activity resides in the methanol extract (STa), whereas the older pig and rabbit ligated loop activity (STb) resides in the methanol-insoluble material. Strain B44 (calf strain) produces STa; this toxin is active in the baby mouse model and calves and is methanol soluble. This is probably typical of calf strains (30). Strain P307 produces STb only, as possibly do a number of infant mouse negative "LT only" strains. The fact that *E. coli* P16 secretes more than one toxin has already been shown (18). Hence, we suggest that the class I

enteropathogens of Moon and Whipp (20) would secrete both STa and STb, and the class II enteropathogens would secrete only STa.

We have found that the ability of *E. coli* strains to secrete STa toxin is occasionally lost on subculturing in vitro; this agrees with other workers (3, 25, 29), but we have so far found the ability to secrete STb to be stable.

We therefore believe that enteropathogenic *E. coli* can secrete two distinct STs, STa and STb. The infant mouse assay is specific for only one of these toxins (STa) and will fail to identify enteropathogenic *E. coli* which produce only STb. Consequently, we recommend that the absence of ST production by enteropathogenic *E. coli* strains should not be based solely on the mouse test but should be based on a combination of the infant mouse test and the rabbit ligated loop assay.

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LITERATURE CITED

1. **Bertschinger, H. U., H. W. Moon, and S. C. Whipp.** 1972. Association of *Escherichia coli* with the small intestinal epithelium. II. Variations in association index and the relationship between association index and enterosorption in pigs. Infect. Immun. **5**:606–611.
2. **Bywater, R. J.** 1972. Dialysis and ultrafiltration of a heat-stable enterotoxin from *Escherichia coli*. J. Med. Microbiol. **5**:337–343.
3. **Dean, A. G., Y. C. Ching, R. G. Williams, and L. B. Harder.** 1972. Test for *Escherichia coli* enterotoxin using infant mice: application in a study of diarrhoea in children in Honolulu. J. Infect. Dis. **125**:407–411.
4. **Dobrescu, L., and C. Huygelen.** 1973. Susceptibility of the mouse intestine to heat-stable enterotoxin produced by enteropathogenic *Escherichia coli* of porcine origin. Appl. Microbiol. **26**:450–457.
5. **Echeverria, P. D., C. P. Chang, and D. Smith.** 1976. Enterotoxigenicity and invasive capacity of 'enteropathogenic' serotypes of *Escherichia coli*. J. Pediatr. **89**:8–10.
6. **Etkin, S., and S. L. Gorbach.** 1971. Studies on enterotoxin from *Escherichia coli* associated with acute diarrhoea in man. J. Lab. Clin. Med. **78**:81–87.
7. **Evans, D. G., D. J. Evans, and N. F. Pierce.** 1973. Differences in the response of rabbit small intestine to heat-labile and heat-stable enterotoxins of *Escherichia coli*. Infect. Immun. **7**:873–880.
8. **Gianella, R. A.** 1976. Suckling mouse model for detection of heat-stable *Escherichia coli* enterotoxin: characteristics of the model. Infect. Immun. **14**:95–99.
9. **Gross, R. J., S. M. Scotland, and B. Rowe.** 1976. Enterotoxin testing of *Escherichia coli* causing epidemic infantile enteritis in the U.K. Lancet **i**:629–631.
10. **Guerrant, R. L., R. A. Moore, P. M. Kirschenfeld, and M. A. Sande.** 1975. Role of toxigenic and invasive bacteria in acute diarrhea of childhood. N. Engl. J. Med. **293**:567–573.
11. **Gyles, C. L., and D. A. Barnum.** 1969. A heat-labile enterotoxin from strains of *Escherichia coli* enteropathogenic for pigs. J. Infect. Dis. **120**:419–426.
12. **Gyles, C. L., J. B. Stevens, and J. A. Craven.** 1971. A study of *Escherichia coli* strains isolated from pigs with gastrointestinal disease. Can. J. Comp. Med. **35**:258–266.
13. **Jacks, T. M., and B. J. Wu.** 1974. Biochemical properties of *Escherichia coli* low-molecular-weight, heat-stable enterotoxin. Infect. Immun. **9**:342–347.
14. **Klipstein, F. A., C.-S. Lee, and R. A. Engert.** 1976. Assay of *Escherichia coli* enterotoxins by in vivo perfusion in the rat jejunum. Infect. Immun. **14**:1004–1010.
15. **Kohler, E. M.** 1968. Enterotoxic activity of filtrates of *Escherichia coli* in young pigs. Am. J. Vet. Res. **29**:2263–2274.
16. **Lariviére, S., and R. Lallier.** 1975. *Escherichia coli* strains isolated from diarrheic piglets in the province of Quebec. Can. J. Comp. Med. **40**:190–197.
17. **Larsen, J. L.** 1976. Differences between enteropathogenic *Escherichia coli* strains isolated from neonatal *E. coli* diarrhoea (N.C.D.) and post weaning diarrhoea (P.W.D.) in pigs. Nord. Veterinaer. Med. **28**:417–429.
18. **Mitchell, I. de G., M. J. Tame, and R. Kenworthy.** 1974. Separation and purification of enterotoxins from a strain of *Escherichia coli* pathogenic for pigs. J. Med. Microbiol. **7**:439–450.
19. **Mitchell, I. de G., M. J. Tame, and R. Kenworthy.** 1974. Conditions for the production of *Escherichia coli* enterotoxin in a defined medium. J. Med. Microbiol. **7**:395–400.
20. **Moon, H. W., and S. C. Whipp.** 1970. Development of resistance with age by swine intestine to effects of enteropathogenic *Escherichia coli*. J. Infect. Dis. **122**:220–223.
21. **Moon, H. W., S. C. Whipp, G. W. Engström, and A. L. Baetz.** 1970. Response of the rabbit ileal loop to cell-free products from *Escherichia coli* enteropathogenic for swine. J. Infect. Dis. **121**:182–187.
22. **Mullan, N. A., M. N. Burgess, and P. M. Newsome.** 1978. Characterization of a partially purified, methanol-soluble heat-stable *Escherichia coli* enterotoxin in infant mice. Infect. Immun. **19**:779–784.
23. **Nalin, D. R., J. C. McLaughlin, M. Rahaman, M. Yunus, and G. Curlin.** 1975. Enterotoxigenic *Escherichia coli* and idiopathic diarrhoea in Bangladesh. Lancet **ii**:1116–1119.
24. **Pierce, N. F., and C. K. Wallace.** 1972. Stimulation of jejunal secretion by a crude *Escherichia coli* enterotoxin. Gastroenterology **63**:439–448.
25. **Raska, K., and H. Raskova.** 1976. Recognizing epidemic strains of *E. coli*. Lancet **i**:1300.
26. **Sack, D. A., J. C. McLaughlin, R. B. Sack, F. Ørskov, and I. Ørskov.** 1977. Enterotoxigenic *Escherichia coli* isolated from patients at a hospital in Dacca. J. Infect. Dis. **135**:275–279.
27. **Sack, D. A., M. M. Merson, J. C. Wells, R. B. Sack, and G. K. Morris.** 1975. Diarrhoea associated with heat-stable enterotoxin producing strains of *Escherichia coli*. Lancet **ii**:239–241.
28. **Sack, R. B., D. A. Sack, I. J. Mehlman, F. Ørskov, and I. Ørskov.** 1977. Enterotoxigenic *Escherichia coli* isolated from food. J. Infect. Dis. **135**:313–317.
29. **Shore, E. G., A. G. Dean, K. J. Holik, and B. R. Davis.** 1974. Enterotoxin-producing *Escherichia coli* and diarrheal disease in adult travellers: a prospective study. J. Infect. Dis. **129**:577–582.
30. **Sivaswamy, G., and C. L. Gyles.** 1976. The prevalence of enterotoxigenic *Escherichia coli* in the feces of calves with diarrhea. Can. J. Comp. Med. **40**:241–246.
31. **Smith, H. W., and S. Halls.** 1967. Observations by the ligated intestinal segment and oral inoculation method on *E. coli* infections in pigs, calves, lambs and rabbits. J. Pathol. Bacteriol. **93**:499–529.
32. **Smith, H. W., and S. Halls.** 1967. Studies on *Escherichia coli* enterotoxin. J. Pathol. Bacteriol. **93**:531–543.

33. **Smith, H. W., and M. A. Linggood.** 1972. Further observations of *Escherichia coli* enterotoxins with particular regard to those produced by atypical piglet strains and by calf and lamb strains: the transmissible nature of these enterotoxins and of a K antigen possessed by calf and lamb strains. J. Med. Microbiol. **5**:243–250.

34. **Söderlind, O.** 1973. Studies on *Escherichia coli* in pigs. IV Reactions of *Escherichia coli* strains in the ligated intestine test. Zentr. Veterinaermed. **20**:558–571.

35. **Whipp, S. C., H. W. Moon, and N. C. Lyon.** 1975. Heat-stable *Escherichia coli* enterotoxin production in vivo. Infect. Immun. **12**:240–244.