INFECTION AND IMMUNITY, July 1976, p. 95-99

Vol. 14, No. 1
Printed in U.S.A.

Suckling Mouse Model for Detection of Heat-Stable *Escherichia coli* Enterotoxin: Characteristics of the Model

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Received for publication 8 March 1976

Although the suckling mouse assay is widely used for the detection of heat-stable *Escherichia coli* enterotoxin (ST), few data have been published concerning the reproducibility, optimal growth, and test conditions of this assay. Four strains of toxigenic *E. coli* known to elaborate both heat-labile enterotoxin and ST or ST alone were used to study these parameters. ST activity after heat treatment and the effect of purified choleragen were also examined. ST production was optimal in Casamino Acids-yeast extract media, but both Trypticase soy and brain heart infusion broths resulted in several false negative reactions. Growing cultures in roller tubes was the most reliable method of ST production. Shaking-flask cultures and stationary-grown cultures resulted in suboptimal ST production in several strains. Optimal mouse incubation time was 3 h, and fluid secretion did not rise thereafter. Adequate toxin production occurred after 16 to 24 h of incubation. The coefficient of variation of various toxins tested on many occasions varied between 10.5 and 15.7%. Toxin activity was stable for 6 months when frozen at −20 C. There was no decrease in ST activity when heated at 65 C for 15 min, but a small decrease was observed in two of four strains after heating at 100 C for 30 min. Choleragen, tested at various doses and at multiple times, gave uniformly negative results. These studies indicate that when done under the proper conditions, the suckling mouse assay is a simple, rapid, and reproducible assay for *E. coli* ST.

Various studies indicate that enteropathogenic *Escherichia coli* cause diarrheal disease either by invasion of the intestinal mucosa or by the elaboration of enterotoxins (1, 5, 8, 18). The enterotoxigenic strains elaborate a high-molecular-weight, heat-labile enterotoxin (LT) or a low-molecular-weight, heat-stable enterotoxin (ST), or both (1, 8, 18). Although several convenient and sensitive assay systems are available for the detection of LT (4, 9), most laboratories dealing with ST rely on the suckling mouse model originally described by Dean et al. (3). In spite of the wide-spread use of this model, a systematic study demonstrating the reproducibility, optimal growth, and test conditions has not been published. It is the purpose of this study to describe this model and to examine many of the variables involved in this assay system with the goals of characterizing the model and describing the optimal conditions for its use.

MATERIALS AND METHODS

Organisms. Five strains of *E. coli* were used in these studies, one toxin-negative strain and four strains producing ST alone or both LT and ST. The characteristics and source of these strains are shown in Table 1. The toxin-producing properties of these strains have been documented in various laboratories (3-5, 10, 17). Strains HS, 334, and B7A were kindly supplied by Sam Formal, Walter Reed Army Institute of Research, Washington, D.C.; strain B44 by Stanley Falkow, University of Washington School of Medicine, Seattle; and strain $214C_1$ by Michael Merson, Center for Disease Control, Atlanta, Ga.

Animals. Newborn Swiss albino suckling mice (1 to 3 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 0.1 ml of a crude culture filtrate containing 2 drops of 2% Evans blue per ml as described by Dean et al. (3) At various times after inoculation, the mice were killed by cervical dislocation, the abdomen was opened, and the entire intestine (not including the stomach), was removed with forceps. The intestines from each group of three mice were pooled and weighed, and the ratio of gut weight to remaining carcass weight was calculated. Animals with no dye in the intestine or with dye within the peritoneal cavity at autopsy were discarded.

Preparation of crude culture filtrates. *E. coli* strains were inoculated into Trypticase soy (TS), brain heart infusion (BHI), or Casamino Acids-yeast extract-salts (CA-YE) broth (7) and incubated at 37 C in a rotary shaking water bath, in a tissue

culture roller tube apparatus, or in stationary cultures for various periods of time. Initially, the culture broths were centrifuged and the supernatant was filtered through a membrane filter (Millipore Corp.) with a pore size of 0.45 μm before testing. Subsequent studies revealed that filtered and unfiltered supernatants behaved identically; therefore the bulk of our studies utilized unfiltered supernatant.

RESULTS

Effect of media and growth conditions. CA-YE medium was the most reliable for ST production (Table 2). BHI medium proved as good as CA-YE for two of the three test strains but inferior with strain 214C_1. TS broth was grossly unreliable, giving negative results with three of the four positive test strains tested.

TABLE 1. *Characteristics of test strains*

Strain	Enterotoxin elaborated	Source
HS	None	Normal volunteer
B44	ST	Diarrheal calf
214C_1	ST	Diarrheal patient, Mexico
344	ST and LT	Diarrheal patient, Calcutta
B7A	ST and LT	Diarrheal patient, Viet Nam

CA-YE cultures grown in roller tubes or shaken flasks, with the exception of strain 214C_1, were equivalent in terms of ST production (Table 2). Stationary cultures were devoid of ST activity.

Because of these findings, all subsequent experiments were performed by using cultures grown in CA-YE medium in roller tubes.

Effect of duration of incubation. Toxin activity was clearly detectable as early as 8 h after inoculation (Table 3). Toxin activity of undiluted samples plateaued at 24 h, with no further significant increase thereafter. Thus, for practical purposes, an incubation time of 24 h seems optimal.

Effect of mouse incubation time. ST-producing *E. coli* grown in CA-YE medium in roller tubes for 24 h resulted in detectable fluid secretion after as little as 1 h (Table 4). The magnitude of fluid secretion reached a plateau at 2 to 3 h and did not significantly increase thereafter. In fact, with two of the strains, B44 and 334, fluid secretion seemed to decrease after 3 to 4 h.

Effect of mouse incubation temperature. The temperature of the environment was critical to a sensitive and reproducible result (Table 5). Optimal temperature was room temperature, approximately 25 C. When animals were kept at 37 C, assay results were either negative

TABLE 2. *Effect of media and growth conditions on toxin activity*[a]

Strain	Medium[b]			Growth conditions[c]		
	TS	BHI	CA-YE	Stationary	Shaker	Roller
B44	0.061 ± 0.002	0.073 ± 0.004	0.115 ± 0.006	0.077 ± 0.005	0.117 ± 0.005	0.111 ± 0.006
214C_1	0.056 ± 0.002	0.074 ± 0.006	0.127 ± 0.009	0.060 ± 0.001	0.071 ± 0.007	0.127 ± 0.009
334	0.118 ± 0.006	0.129 ± 0.009	0.115 ± 0.003	0.069 ± 0.010	0.110 ± 0.013	0.103 ± 0.005
B7A	0.058 ± 0.003	0.127 ± 0.006	0.152 ± 0.004	0.072 ± 0.004	0.137 ± 0.008	0.152 ± 0.008

[a] Figures represent mean ± 1 standard error of the mean of five separate assays of toxin preparations grown in various media under various culture conditions for 24 h.
[b] Grown in 5 ml of broth in roller tubes.
[c] Stationary, Unagitated flask; shaker, in flask in rotary shaker water bath at 120 rpm; roller, roller test tubes at 2 rpm. All grown in CA-YE medium.

TABLE 3. *Effect of duration of incubation on toxin activity*[a]

Strain	Time (h)							
	8		16		24		48	
334	0.125 ± 0.006	NS[b]	0.127 ± 0.005	NS	0.136 ± 0.004	NS	0.150 ± 0.007	
B7A	0.104 ± 0.006	<0.02	0.133 ± 0.007	<0.05	0.152 ± 0.004	NS	0.149 ± 0.016	

[a] Grown in CA-YE medium in roller tubes. Mouse incubation period, 4 h. Figures are mean ± 1 standard error of the mean of five separate assays at each time period.
[b] NS, Not significant. *P* values refer to comparison of times, i.e., 8 h versus 16 h, etc.

TABLE 4. *Effect of mouse incubation time on toxin activity*[a]

Strain	Time (h)					
	1	2	3	4	6	8
HS	0.057 ± 0.010	0.059 ± 0.007	0.060 ± 0.004	0.060 ± 0.007	0.058 ± 0.007	0.051 ± 0.006
B44	0.089 ± 0.007	0.102 ± 0.004	0.111 ± 0.006	0.096 ± 0.007	0.090 ± 0.006	0.080 ± 0.008
214C$_1$	0.097 ± 0.003	0.116 ± 0.013	0.127 ± 0.009	0.105 ± 0.006	0.107 ± 0.007	0.119 ± 0.009
334	0.104 ± 0.016	0.125 ± 0.007	0.126 ± 0.005	0.126 ± 0.008	0.094 ± 0.003	0.095 ± 0.005
B7A	0.098 ± 0.008	0.128 ± 0.009	0.142 ± 0.008	0.135 ± 0.001	0.130 ± 0.009	0.133 ± 0.008

[a] Grown in CA-YE medium for 24 h in roller tubes. Figures are mean ± 1 standard error of the mean of five separate assays.

TABLE 5. *Effect of mouse incubation temperature*[a]

Strain	25 C	37 C
334	0.130 ± 0.004	0.076 ± 0.004[b]
B7A	0.131 ± 0.005	0.107 ± 0.005[b]

[a] Grown in CA-YE medium for 24 h in roller tubes. Mouse incubation time is 3 h. Figures are mean ± 1 standard error of the mean of five separate assays.

[b] $P < 0.01$.

(strain 334) or markedly inhibited (strain B7A).

Reproducibility of the suckling mouse assay. This assay system was reliable and reproducible (Table 6). The coefficient of variation of the toxin activity of various strains tested on many different occasions varied between 10.5 and 15.7%, whereas that of the same strain tested 10 times on the same day was 8.9%.

On the basis of the strains we have examined and using 95% confidence intervals, gut weight/carcass weight ratios less than 0.074 are considered negative, those 0.075 to 0.082 are considered indeterminate and should be repeated, and those greater than 0.083 are considered positive. These values are comparable to those reported by other investigators (3, 10, 12, 17, 20).

Stability of toxin activity. Strain B7A was grown in CA-YE medium in roller tubes for 24 h, and aliquots of the supernatant were frozen at −20 C. Periodically, aliquots were defrosted and tested. Toxin activity was stable for at least 6 months when stored in this fashion, i.e., activity of freshly prepared ST 0.134 ± 0.006 (n = 10) versus 6-month-frozen toxin 0.135 ± 0.005 (n = 10).

Effect of heat on toxin activity. Because of the lack of a standardized definition of ST, the effect of two heat treatments on suckling mouse activity was examined (Table 7). There was no decrease in toxin activity in any of the four toxins tested when they were heated at 65 C for 15 min. In contrast, a small but significant decrease in toxin activity was observed when toxins from strains B44 and 334 were heated at 100 C for 30 min.

Effect of pure cholera toxin. Purified cholera toxin (kindly supplied by Carl Miller, Cholera Research Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md.) was tested in this model (Table 8). Doses of purified choleragen from 0.1 to 10.0 μg per mouse gave uniformly negative results at all incubation times from 2 to 8 h.

DISCUSSION

Our results indicate that, under the proper conditions, the suckling mouse assay is a reproducible assay system for the detection of heat-stable *E. coli* enterotoxin. Sack et al. (17) have shown that the suckling mouse assay correlates well with the 6-h in vivo rabbit ileal loop assay for ST (6). In our hands, the day-to-day variability of the suckling mouse assay ranges from 10.5 to 15.7%, depending upon the strain. Of the three growth media tested, CA-YE medium (7) seems to be the most reliable for ST elaboration, since all four of the known ST-producing test strains produced ST in this medium. In contrast, three of four test strains were negative when grown in TS broth, and two of four were negative when grown in BHI broth. In addition, CA-YE medium is more economical than either TS or BHI broth.

Growing a roller tube culture for 16 to 24 h reliably resulted in ST elaboration. In contrast, stationary cultures were totally unsuitable, all four positive test strains being negative when grown in this medium. Shaker-flask cultures produced comparable ST activity with three of the four test strains but was negative with a fourth. Because of this last observation and the ease of handling large numbers of roller tubes, i.e., screw-cap test tubes (16 by 125 mm) containing 5 ml of medium, we strongly recommend the use of the roller tube method.

Although *E. coli* ST results in intestinal secretion as early as 1 h, a characteristic of ST noted by others in other model systems (2, 6) and in contrast to LT, 3 to 4 h seems to be required for maximal intestinal fluid secretion. Longer mouse incubation times give unpredict-

TABLE 6. *Reproducibility of the suckling mouse assay*[a]

Strain	Determination				
	No. of assays	Mean	2 SD[b]	CV[c] (%)	95% CI[d]
HS	21	0.060	0.014	11.7	0.046–0.074
B44	15	0.106	0.024	11.5	0.082–0.130
$214C_1$	10	0.126	0.023	13.1	0.103–0.149
334	14	0.130	0.028	10.5	0.102–0.158
B7A	14	0.141	0.044	15.7	0.097–0.185

[a] Grown in CA-YE medium for 24 h in roller tubes. Mouse incubation period; 4 h.
[b] Standard deviation.
[c] Coefficient of variation.
[d] 95% Confidence intervals.

TABLE 7. *Effect of heat on toxin activity*[a]

Strain	No heat	65 C, 15 min	100 C, 30 min
B44 (5)	0.096 ± 0.007	0.097 ± 0.010	0.081 ± 0.008[b]
$214C_1$ (5)	0.127 ± 0.009	0.125 ± 0.006	0.117 ± 0.004
334 (6)	0.129 ± 0.007	0.128 ± 0.005	0.103 ± 0.007[c]
B7A (8)	0.118 ± 0.010	0.122 ± 0.010	0.104 ± 0.012

[a] Grown in CA-YE medium for 24 h in roller tubes. Mouse incubation period, 3 h. All figures are mean ± 1 standard error of the mean. Figures in parentheses represent number of assays.
[b] $P < 0.05$ compared with "No heat."
[c] $P < 0.01$ compared with "No heat."

able results, with some strains maintaining maximal gut secretion for up to 8 h, whereas with other strains fluid secretion seems to decline after 3 to 4 h. We now routinely use the 3-h incubation period since, with proper staggering of time of inoculation, two to three large assay groups and a large number of strains can be comfortably screened in an 8-h work day.

The environmental temperature in which the mice are kept after inoculation can strongly influence the test result. As shown in Table 5, the animals should be kept at room temperature, approximately 25 C, since our results indicate that an environmental temperature of 37 C will result in false negative or weakly positive results.

Of the four crude preparations of ST examined, none were affected by heating at 65 C for 15 min. This is consistent with the reports of other investigators (11, 15). In contrast, a small but significant decrease in toxin activity was observed in two of four strains after heating at 100 C for 30 min. Several authors have reported that *E. coli* ST is resistant to heating at 100 C for 30 min (6, 16, 17), whereas others (10, 12, 20) have reported a marked decrease in toxin activity with this treatment. These reported differences may be due to differences in strains, growth media, and/or the methods used. This explanation is consistent with our own observations that two strains were somewhat heat sensitive whereas two other strains were totally heat resistant.

These observations also raise the possibility that our current definition of *E. coli* ST, i.e., a low-molecular-weight, heat-resistant substance, may not define a single species, but that these strains may elaborate more than a single substance with ST activity. This view has been stated by others (2, 14, 19) and is consistent with the different estimates of ST molecular weights reported by a variety of investigators (2, 11–14).

Previous authors have stated that the suckling mouse model is relatively specific for ST (3, 12, 17). To further examine this question, various doses of purified choleragen were tested in this model. Doses of 0.1 to 10.0 μg per mouse gave uniformly negative results even when tested for as long as 8 h. Similar results were obtained by Dean et al. (3), using crude cholera toxin. The fact that choleragen and *E. coli* LT are thought to be similar molecules, i.e., demonstrating immunological cross-reactivity and many physiological parameters in common (1, 18), would suggest that the suckling mouse model does not react to *E. coli* LT. In fact, Jacks and Wu (12) and Whipp et al. (20) have demonstrated that the suckling mouse model does not respond to inoculation with preformed, albeit only semipurified, *E. coli* LT. These results, taken in toto, strongly suggest that the suckling mouse assay is at least relatively specific, if not specific, for ST. A definitive answer to this question must wait the availability of pure *E. coli* LT.

In summary, our studies indicate that if done under the proper conditions, the suckling mouse assay is a simple, rapid, and reproducible assay system for *E. coli* ST. Furthermore, our data, as well as those of others, indicate

TABLE 8. *Effect of cholera toxin on suckling mouse assay*[a]

Dose (μg)	Time (h)			
	2	4	6	8
0.1	0.064 ± 0.003	0.059 ± 0.003	0.053 ± 0.004	0.055 ± 0.003
1.0	0.074 ± 0.005	0.070 ± 0.005	0.071 ± 0.002	0.067 ± 0.002
10.0	0.069 ± 0.003	0.059 ± 0.002	0.070 ± 0.003	0.066 ± 0.003

[a] Each timepoint represents the mean ± 1 standard error of the mean of five tests.

that this model primarily detects ST and that some forms of ST are, to some degree, heat sensitive.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Service research contract 1-AI-4-2549 from the National Institute of Allergy and Infectious Diseases and by VA research project 596-3108-01.

I am indebted to Lauren Brasile for expert technical assistance.

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