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AGE-DEPENDENT VARIATION IN THE DENSITY AND AFFINITY OF *ESCHERICHIA COLI* HEAT-STABLE ENTEROTOXIN RECEPTORS IN MICE

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1. SUMMARY

Enterotoxigenic strains of *Escherichia coli* that produce heat-stable enterotoxin (STa), are a major cause of diarrheal disease worldwide. Resistance to diarrheal disease in human infants and newborn animals has been attributed to a gradual turnover in the intestinal brush border membrane receptors to bacterial pili. In this study, we demonstrated age-dependent variation in the density and affinity of the mouse enterocyte receptors specific for STa. Flow cytometry and radiolabeled-STa (^{125}I-STa) assays were used as more reliable quantitative measures for the characterization of STa-enterocyte receptor interaction. These assays indicated a stronger interaction of STa with its putative receptor on the enterocytes of the 2-day-old suckling mice than with enterocytes from 1-week, 2-week and 2-month-old mice. Scatchard plot analysis of ^{125}I-STa-receptor interaction suggested that STa-receptors exist at a higher number on enterocytes from the 2-day-old mice than enterocytes of the older mice. Additionally, receptors from the 2-day-old mice had a greater affinity for STa ligand than receptors from the older mice. Density of STa receptors on enterocytes and their affinity to STa may determine the extent of binding and severity of secretory response. This may further explain the increased susceptibility of newborn animals and human infants to STa-mediated diarrheal disease.

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2. INTRODUCTION

Secretory diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) is a major cause of death among human infants and young animals in the developing countries (Dreyfus et al., 1984; Saeed et al., 1987; Vaandrager et al., 1993). Virulence factors that enable ETEC strains to cause diarrheal disease during the first days of life include specific surface fimbriae, which mediate bacterial adherence to intestinal epithelial cells, and enterotoxins that stimulate intestinal secretion (Butler and Clarke, 1994; Jaso-Friedmann et al., 1992; Sack, 1980). It was found that colonization by ETEC can be blocked either by lack of fimbrial receptors, or presence of receptor analogs in the mucous layer that inhibit binding to intestinal cells suggesting a key role of ETEC fimbriae in host specificity and age susceptibility in pigs (Dean, 1990). ETEC produce different types of enterotoxins; heat-labile enterotoxin (LT) and two types of heat-stable enterotoxins (STa and STb) (Saeed and Greenberg, 1985; Sears and Kaper, 1996). STa-mediated diarrhea is more common and more severe in young animals and human infants (Cohen et al., 1986; 1988; Rao et al., 1981; Saeed et al., 1987). STa is a cysteine-rich, nonimmunogenic, 18- or 19-amino acid peptide with a molecular weight of 2 kDa (Butler and Clarke, 1994; Sears and Kaper, 1996). STa has been found to increase guanylate cyclase activity and guanosine 3′,5′-cyclic monophosphate (cGMP) concentration in the mammalian small intestinal cells (Guandalini et al., 1982; Guerrant et al., 1980). The sequence of events which ends in stimulation of intestinal fluid secretion and diarrhea is initiated by STa binding to a specific receptor located on the brush border membrane of the intestinal epithelial cells of the host (Giannella et al., 1983; Jaso-Friedmann et al., 1992). STa receptor is believed to be part of the extracellular motif of the brush border-associated guanylyl cyclase (Schulz et al., 1990; Vaandrager et al., 1993; Wada et al., 1994). The STa/receptor binding has been studied in human, pig and rat intestine. In all of these species, an increase in brush border membrane-STa receptor density was observed in the immature intestine (Cohen et al., 1986; 1988; Jaso-Friedmann et al., 1992). This coincides with the period of increased susceptibility to STa-induced diarrheal disease that occurs in early life of humans and animals. Stevens et al. (1971). have described two periods of increased porcine responsiveness to STa; during the first week of life and directly after weaning. It is not clear whether the susceptibility to ETEC-STa changes with age and if this change results from alterations in the density and/or affinity of the enterocytes receptors that are specific for this enterotoxin. The development of age-dependent resistance against ETEC diarrheal diseases was observed in more than one species of animals. Moon and Whipp (1970) found that STb is capable of inducing secretion in 7-day-old pigs but not in 7-week-old ones. Previous studies on the effect of age on the interaction between STa and its putative receptor revealed that immature rat jejunum was much more sensitive to the secretory effect of STa than adult jejunum. In this study, we hypothesized that the susceptibility of the 2-day-old mice that are used in the suckling mouse assay of ETEC-STa is modulated by an increased number of STa receptors on their enterocytes, and that the affinity of these receptors to the STa toxin may be age-dependent. Currently, the suckling mouse model is the only reliable bioassay for ETEC-STa. Flow cytometry, ^{125}I-STa affinity binding and indirect immunofluorescence assays were utilized to characterize the interaction of ETEC-STa with its putative receptor on enterocytes of different age-groups of mice.

3. MATERIALS AND METHODS

3.1. STa Purification

STa was produced and purified to homogeneity using the methods described by Staples et al. (1980) and modified by Saeed and Greenberg (1985).

3.2. Experimental Animals

Four different age groups (2-day, 1-week, 2-week, and 2-month-old) of Swiss Webster mice were used in this experiment (8–10 mice in each group). The mouse room had 13–15 complete air changes per hour and was maintained at 22 ± 1°C temperature with 45 ± 2% relative humidity and a 12/12-h light/dark cycle. Mice were euthanized by ether anesthesia followed by cervical dislocation, and single cell suspensions of enterocytes were prepared from each group as described below.

3.3. Isolation of Suckling Mouse Enterocytes

Enterocytes were isolated as described previously (Al-Majali et al., 1998). The population of cells harvested was monitored by periodic wet mount examination through the whole procedure to assess the quantity and quality of the isolated enterocytes. Cell counts and cell viability were determined by dye-exclusion using 0.2% trypan blue. Only cell suspensions that contained over 80% viable cells were used for indirect immunofluorescence, flow cytometric analysis and ^{125}I-STa binding assay.

3.4. Indirect Immunofluorescence Assay

Intestinal cryostat sections were incubated with 50µl (100µg/ml of 10mM PBS) of HPLC-purified STa for 45 minute at 37°C. After washing three times with PBS (pH 7.4), slides were incubated at 37°C for 45 minute with 50µl of 1:10 diluted anti-STa antibody produced in rabbit. Slides were washed three times in PBS and reincubated with 50µl of 1:100 diluted anti-rabbit-IgG-FITC-conjugated antibody (KPL, Gaithersburg, MD). After a 45-minute incubation, slides were rinsed in PBS and examined using a Nikon labophot epi-fluorescence microscope.

3.5. Flow Cytometry Analysis

Enterocytes were prepared for staining by three additional washes with PBS, pH 7.2, containing 0.5% BSA. In a volume of 100µl, 10^5 enterocytes in PBS-BSA were incubated with 50µl of HPLC purified STa (10µg/ml of 10mM PBS) for 45 minute at 37°C. After washing three times in PBS-BSA, enterocytes were resuspended in 100µl of PBS-BSA. Fifty-microliter of STa-specific antiserum produced in rabbits was diluted 1:10 in PBS, added to the enterocyte suspension and incubated for 30 minute at 4°C. Cells were washed three times with PBS-BSA and resuspended in 100µl of PBS-BSA. Fifty-microliter of goat anti-rabbit-IgG-FITC-conjugated antibody (KPL, Gaithersburg, MD) diluted 1:100 in PBS was added to the enterocyte suspension and incubated for 30 minutes on ice. Cells were washed three times with PBS-BSA, resuspended in 1.0ml of PBS, and kept on ice until flow cytometric analysis was performed. As

a negative control, similar samples were incubated only with the secondary FITC-conjugated antibody and used to determine the threshold of specific staining. Flow cytometric analysis was performed using the Epics ELITE flow cytometer (Coulter Electronics, Hileah, FL.). FITC-stained cells were excited by using 15 mW of 488 nm argon laser light. Calibration beads were run and the mean fluorescent intensity was set at a fixed value, which was maintained throughout the experiment.

3.6. STa Iodination

HPLC-purified STa was radioiodinated in a reaction mixture that contained the following: STa, 100 µg; 0.2 M sodium phosphate (pH 7.2), 45 µl; one Iodo-bead® (Pierce, Rockford, IL); Na-^{125}I (NEN, Boston, MA), 1.0 mCi; and 2% D-glucose, 25 µl. After 15 minutes incubation at room temperature, radiolabeled STa (^{125}I-STa) was separated from free iodine using a Sep-Pack C-18 cartridge column (Waters Associates, Milford, MA). The column was pre-washed with 10 ml 100% methanol and equilibrated with 10 ml distilled water. Stepwise elution of the ^{125}I-STa was performed with (i) 10 ml of 0.1% trifluoroacetic acid (TFA) in 30% methanol (HPLC grade), (ii) 10 ml of 0.1% TFA in 60% methanol eluted, and (iii) 10 ml of 0.1% TFA in 100% methanol.

3.7. Binding Assay

Reaction mixtures containing isolated mice entrocytes (2×10^5), PBS-BSA and ^{125}I-STa (20–640 nM) were incubated in a final volume of 200 µl for 40 minute at 37°C in a shaking water bath. Unbound ^{125}I-STa was removed from bound ^{125}I-STa by vacuum filtration (Millipore Corp., Bedford, MA), using 1-µm, 2.5 cm GF/B glass filters (Whatman, Maidstone, England). Total binding was measured in a reaction mixture that did not contain the unlabeled STa, whereas nonspecific binding was measured in a reaction mixture that contained the labeled STa including 1000-fold excess of unlabeled STa. Specific binding was calculated by subtracting non-specific binding from the total binding. All experimental points were determined in duplicate. Specific binding data were used to calculate the apparent dissociation constants (K_d) and the maximum number of STa receptors (B_{max}) associated with enterocytes (Scatchard, 1949).

4. RESULTS

4.1. Indirect Immunofluorescence Assay

Indirect immunofluorescence study of enterocytes from STa-susceptible mice revealed the localization of intensely stained areas mostly at the brush border membrane region. Cryostat sections obtained from small intestine of 2-day-old suckling mice showed an intensely fluorescent brush border membrane after treatment with rabbit anti-STa and anti-rabbit-IgG-FITC-conjugated antibodies (Fig. 1). Some fluorescence was found in some focal areas inside the mucosa of the intestine. Fluorescence intensities were relatively low in the intestinal sections and enterocyte smears obtained from 1-week and 2-week-old mice when compared with that of the 2-day-old suckling mice suggesting that fluorescence intensity is inversely related to age.

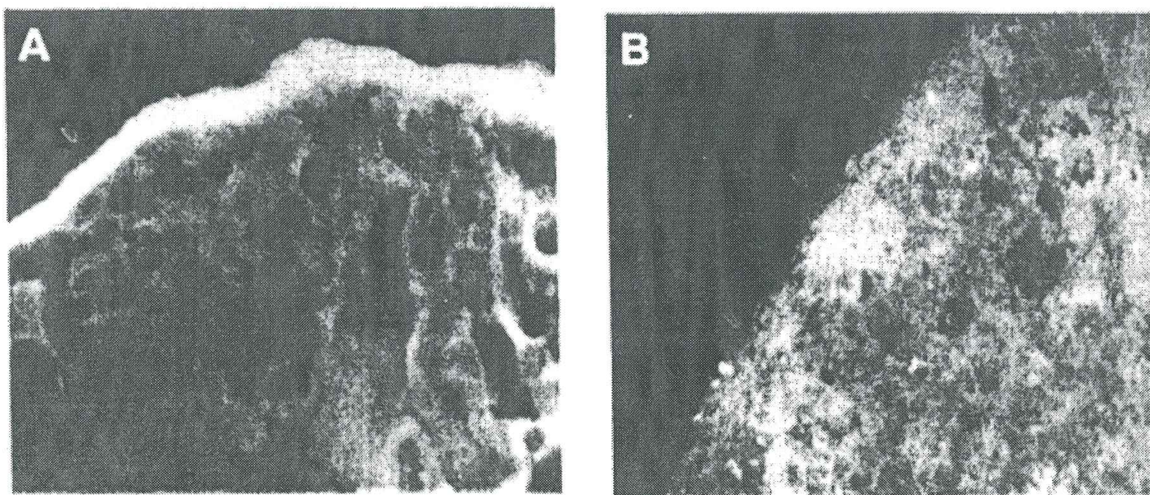

Figure 1. Immunofluorescence staining of STa toxin in cryostat sections of mice small intestine. A: 2-day-old suckling mouse intestinal section incubated with STa and anit-STa-Rabbit IgG serum and stained with FITC-conjugated anti-rabbit IgG antibodies; B: Control, 2-day-old suckling mouse intestinal section incubated only with STa and stained by FITC-conjugated anti-rabbit IgG antibodies. (×1000).

4.2. Flow Cytometry Analysis

The binding of STa to its putative receptor was studied using flow cytometric analysis. A histogram showing significantly increased fluorescence intensity was associated with the 2-day-old suckling mice enterocytes that were stained with rabbit anti-STa and anti-rabbit-IgG-FITC-conjugated antibodies. Only weak fluorescence was demonstrated in similarly prepared and processed samples from the 1-week and 2-week-old mice. No fluorescence was observed on processed enterocytes from any age groups when no STa was added. The staining results of freshly isolated enterocytes from different age groups of suckling mice are shown in Fig. 2.

4.3. Effect of ^{125}I-STa Concentration on Binding

Binding assays with enterocytes from each age group were performed to characterize the association of STa with its putative receptor on the enterocyte surface. The binding of ^{125}I-STa to enterocytes from each group of mice was saturable, and reached a plateau (Fig. 3). The specific binding of ^{125}I-STa to enterocytes from 2-day-old mice was about 2-fold higher than the specific binding of ^{125}I-STa to enterocytes obtained from 1-, 2-week, and 2-month old mice. Non-specific binding in the 2-day-old group enterocytes accounted for only 4–8% of total binding whereas non-specific binding for enterocytes from the 1-week, 2-week, and 2-month old mice accounted for 40–50% of the total binding.

4.4. Stoichiometry of ^{125}I-STa Binding to the Different Age Group of Mice Enterocytes

Scatchard analysis of specific binding data suggest the existence of a single class of STa receptors associated with enterocytes from different age groups. Calculation of dissociation constants (K_d) and maximum number of receptors (B_{max}) suggested higher

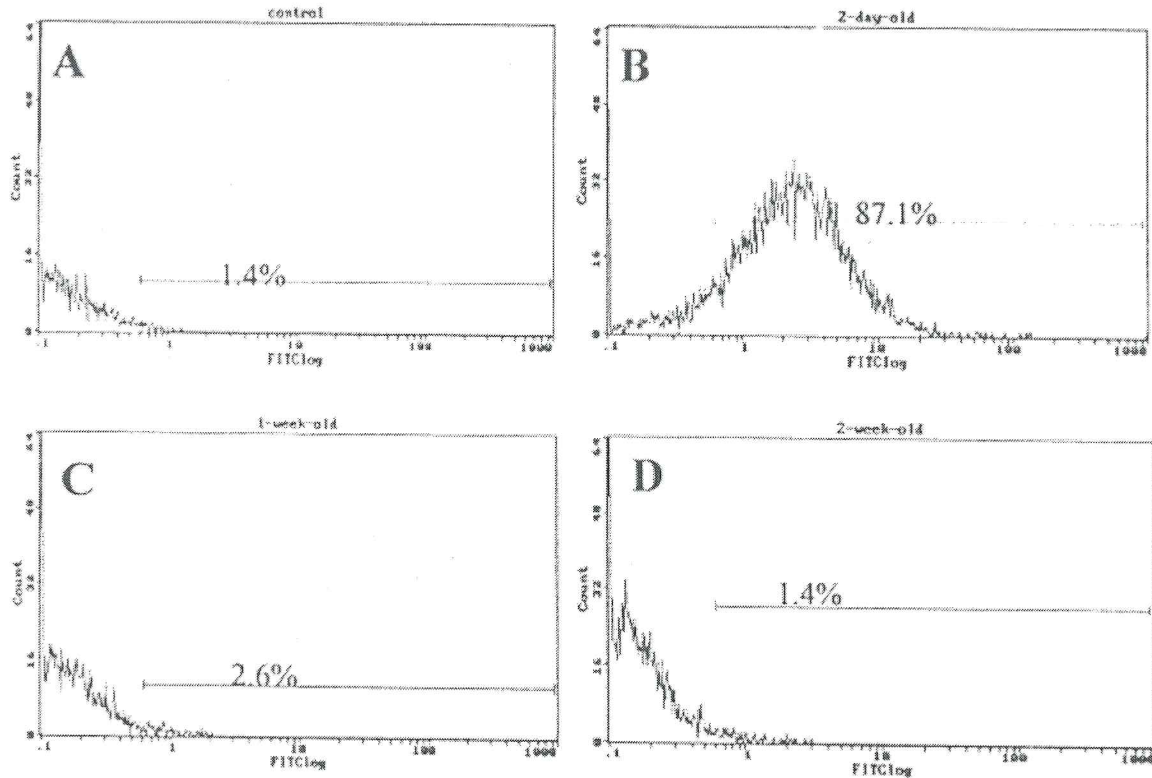

Figure 2. Representative flow cytometric histograms from different age groups of mice. A: control, no STa toxin was added; B: Enterocytes from 2-day-old mice; C: Enterocytes from 1-week-old mice; D: 2-week-old mice. Enterocytes where incubated with STa, rabbit anti-STa serum, and stained with anti-rabbit-IgG-FITC conjugated antibodies. Similar trends were obtained upon repeating the experiment.

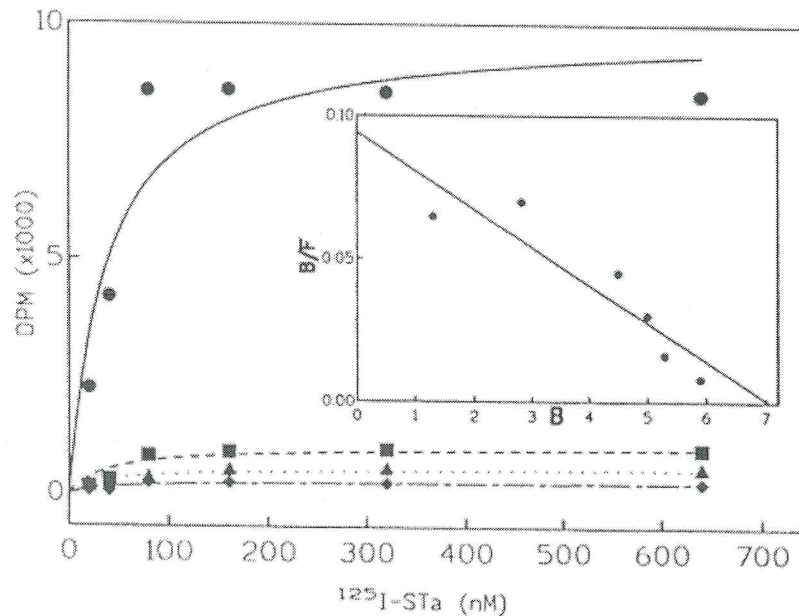

Figure 3. Specific binding of ^{125}I-STa to enterocytes obtained from different age groups of mice in the presence of increasing concentrations of ^{125}I-STa. ●——●, enterocytes from 2-day-old suckling mice; ■- - -■, enterocytes from 1-week-old suckling mice; ▲. . . . . .▲, enterocytes from 2-week-old mice; and ◆—·—◆, enterocytes from adult (2-month-old) mice. Nonspecific binding was determined in incubation mixtures containing ^{125}I-STa and a 1000-fold excess of unlabeled STa. Similar trends were obtained upon repeating the experiment. Inset represents Scatchard plot for the 2-day-old enterocytes. Scatchard plot analyses for the specific binding data of the different age groups are shown in Table 1.

Table 1. Binding properties of ^{125}I-STa to enterocytes from mice of different age groups[a]

Mouse age group	Specific binding (%)[b]	Dissociation constant (nM)	STa receptor density (nM/mg protein)
2-day-old	94	75	7.2
1-week-old	72	125	0.30
2-week-old	62	1430	0.36
2-month-old	60	1111	0.40

[a] Similar trends were obtained upon repeating the experiment.
[b] Each number represents the average of 6 readings.

affinity and receptor density for STa in the 2-day-old suckling mice enterocytes than in other age groups. STa-receptor density of the 2-day-old suckling mice was 20-fold higher than that of the 1-week, 2-week-, and 2-month-old mice (Table 1). The dissociation constant of STa receptor of enterocytes obtained from 2-day-old suckling mice (75 nM) was 10-fold lower than that obtained from 2-week and 2-month-old mice. The K_d of the 1-week-old suckling mice (125 nM) was 2-fold higher than that of the 2-day-old suckling mice. ^{125}I-STa binding properties are shown in Table 1.

5. DISCUSSION

The age-dependent resistance to diarrheal disease caused by enterotoxigenic *Escherichia coli* (ETEC) was first reported in pigs. Moon and Whipp (1970), found that some strains of ETEC cause secretory diarrhea only in neonatal pigs under two weeks old, whereas, other strains have the ability to cause diarrhea in neonatal and older pigs. Enterotoxins are among the most important virulence factor of ETEC and are considered the immediate mediator of diarrhea (Cohen et al., 1988; Sears and Kaper, 1996). High doses of STa were found to affect the secretory response in ligated intestinal loops, and addition of STb to STa-treated loops increased this secretory response. Although differences may exist in the sensitivity of neonatal and adult hosts to bacterial enterotoxins, little is known about changes of enterotoxin receptor affinity and density in the first weeks after birth.

In this study, the presence of STa receptors on enterocytes obtained from mice of different age groups was demonstrated using flow cytometry and indirect immunofluorescence assays. The absence of fluorescence in the control group, where no toxin was added, suggested a specific interaction between STa and its putative receptor. The significant increase in the fluorescence intensity in the 2-day-old suckling mice enterocytes, which were treated with STa, rabbit anti-STa, and anti-rabbit-IgG-FITC conjugated antibodies, must have been due to an increase in either the receptor number or the affinity of STa to these receptors (Fig. 3). Similar results were obtained using indirect immunofluorescence staining (Fig. 1). For further investigation of STa-receptor stoichiometry, ^{125}I-STa binding affinity to STa-receptors on the mice-enterocytes was performed. The ^{125}I-STa binding affinity data suggested that a significantly higher number of STa receptors was present on the enterocytes of the 2-day-old mice group than older mice (Fig. 3). The number of the STa receptors on the enterocytes of the 1-week, 2-week, and 2-month-old mice was significantly lower than that of the 2-day-old mice (Table 1). Unlike previous reports in pigs (Jaso-Friedmann et al., 1992), our data suggests an increase in the STa receptor affinity in the 2-day-old suckling mice (Table 1). This

age-dependent affinity of STa receptors may be due to conformational or structural changes in the extracellular domain of the guanylate cyclase protein. Further investigation is needed to elucidate this age-dependent affinity. Binding of ^{125}I-STa to mice intestinal cells was shown to be rapid, specific, saturable, temperature dependent, and belongs to a single class of receptors. It is unclear why STa receptors exist in a larger number on enterocytes from neonatal animals. It is possible that the STa receptor functions as a receptor for growth promoting peptide(s) and that an increased number of receptors for this peptide would be needed in the intestine of the neonate. It is noteworthy that, recently, we studied the effect of dietary insulin on the response of suckling mice enterocytes to STa. Insulin was found to up-regulate this response (Al-Majali et al., 1998). It is likely that multiple factors contribute to the increased susceptibility of newborn animals to ETEC. This predilection might be variably expressed on the basis of permissive host factors, including brush border membrane changes that may be induced by dietary antigens, stress, or by environmental factors. The high susceptibility to ETEC might be augmented or more fully expressed in response to host or environmental factors. In addition, this report describes the use of flow cytometry to study the interaction of STa with its putative receptor. Conventional fluorescent microscopy analysis results only in inaccurate estimates of fluorescence intensity due to the scoring protocol used (++++; for high fluorescent intensity, +; for relatively low fluorescent intensity). Using flow cytometry, studying the STa/receptor interaction was possible through accurate determination of the intensity of fluorescence on enterocytes of the different age groups of suckling mice. In summary, we have demonstrated using indirect flow cytometry, immunofluorescence, and ^{125}I-STa binding assays that, in suckling mice, STa-receptor numbers and affinity are age-dependent. STa-receptor numbers and affinity were higher in the 2-day-old mice suckling mice than older mice. These results may further explain the increased susceptibility of immature and young animals to STa-mediated diarrheal disease. This model will be utilized in future studies for further investigations of the mechanism of STa-mediated diarrheal disease in humans and animals.

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