

## 27. Heat-stable enterotoxins: properties, detection and assay

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### SUMMARY

The heat-stable enterotoxins (STs) are one of two classes of toxins responsible for diarrheal disease caused by enterotoxigenic strains of *Escherichia coli*. Two distinct classes of these peptide toxins (ST<sub>a</sub> and ST<sub>b</sub>) have been identified. ST<sub>a</sub> has been well characterized whereas ST<sub>b</sub> has received little study. Here, certain aspects of ST<sub>a</sub> and ST<sub>b</sub> are reviewed: chemical structure, behavior toward physical and chemical agents, mode of action, genetics and regulation, detection of *E. coli* strains producing ST, and detection and quantitation of ST.

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### INTRODUCTION

Strains of enterotoxigenic *Escherichia coli* (ETEC) produce diarrhea in man and animals; the disease symptoms are intimately related to ingestion of ETEC-contaminated food or water. ETEC strains produce either an immunogenic heat-labile enterotoxin (LT = labile toxin) of high molecular weight which is similar in structure and mode of action to cholera toxin and/or a second enterotoxin that is a heat-stable nonimmunogenic peptide (ST = stable toxin). Both toxins produce a similar diarrheic disease. Certain aspects of ST structure, chemical behavior, mode of action, detection and assay are reviewed here.

### HEAT-STABLE ENTEROTOXINS (STs) FROM *E. COLI*

#### ST<sub>a</sub>

ST-producing strains of ETEC have been isolated from bovine, porcine, and human hosts [21]. The STs have been associated with cases of travelers' and childhood diarrhea [17] as well as diarrhea in young animals [49].

There are at least two types of ST: ST<sub>a</sub> (STI) and ST<sub>b</sub> (STII). ST<sub>a</sub> is a methanol-soluble peptide which has biological activity in suckling mice and neonatal pigs but is inactive against weaned pigs; ST<sub>b</sub> is methanol-insoluble and has biological activity in ligated ileal loops of weaned pigs but is inactive against suckling mice [8,17]. ST<sub>a</sub> is a poor antigen [4]; antibodies to ST<sub>a</sub> were produced by conjugating purified ST<sub>a</sub> to either bovine immunoglobulin G or bovine serum albumin [21,25]. However, Lallier et al. [40] stated that purified ST<sub>a</sub> could be immunogenic if a sufficient quantity was combined with an adjuvant, but their procedure does not appear to have been used by later workers. ST<sub>a</sub> and ST<sub>b</sub> are immunologically and genetically distinct [21,25,42, 57]. ST<sub>a</sub> (and presumably ST<sub>b</sub>) has no antigenic identity with ETEC LT, cholera toxin, or staphylococcal enterotoxin B [25]. ST<sub>a</sub> from ETEC strains isolated from bovine, porcine, and human sources all cross-react immunologically [21,25].

The ETEC toxins are differentiated on the basis of their heat stability: LT is inactivated when held for 30 min at 60°C whereas ST is stable for several hours at 60°C [38,74]. Takeda et al. [80] demonstrated that 50% of the biological activity of puri-



1B) and found that the analog was two to five times more potent than the complete peptide in the suckling mouse assay. The analog was stable at 120°C for at least 30 min and thus was similar in heat stability to the complete molecule. The studies with ST<sub>a</sub>-18 and ST<sub>a</sub>-19 indicate that some amino acid losses or interchanges have no effect on biological activity but disulfide bridges are essential for activity.

ST<sub>a</sub> molecules cause their diarrheagenic effect by increasing the intracellular concentration of cyclic guanosine 3',5'-monophosphate (c-GMP) of cells in the intestinal mucosa due to increased guanylate cyclase activity [58,60]. Thus, the overall effect of ST<sub>a</sub> is to stimulate fluid and electrolyte secretion in intestinal mucosa; it does not affect other transport systems such as glucose transport [58,60].

The effect of ST<sub>a</sub> is rapid with no lag phase, reversible (by washing the intestine), short-term, and tissue-specific. It is possible that the diarrhea itself may reverse ST<sub>a</sub> action by diluting the toxin from its receptors. ST<sub>a</sub> stimulates guanylate cyclase in various segments of the small and large intestine but not in a variety of other tissues. ST<sub>a</sub> acts only on particulate guanylate cyclase; it has no effect on the soluble enzyme [31,58,60].

Ganguly and Talukder [22] studied the effect of ST<sub>a</sub> on <sup>45</sup>Ca<sup>2+</sup> uptake by rat intestinal brush border membrane vesicles and demonstrated up to five-fold, dose-dependent increase in Ca<sup>2+</sup> uptake. The authors suggested that ST<sub>a</sub> may be acting as a calcium ionophore.

There have been a few studies on the nature of the ST<sub>a</sub> receptor site(s). Frantz et al. [20] and Dreyfus and Robertson [12] found that ST<sub>a</sub> bound to isolated rat intestinal epithelial cells and brush border membranes. The binding of ST<sub>a</sub> to the receptor was irreversible, and was dose- and temperature-dependent. In contrast, Cohen et al. [10] demonstrated that both ST<sub>a</sub> binding to rat brush border membrane receptors and ST<sub>a</sub>-mediated fluid secretion were rapidly and spontaneously reversible. The ST<sub>a</sub>s from porcine, bovine, and human ETEC strains bound to the receptor equally well [20]. Binding was not inhibited by carbohydrates, gangliosides or glycolipids; however, treatment of brush

border membranes with pronase or trypsin inhibited binding [12]. The nature of the binding of ST<sub>a</sub> to the receptor is not known, but since disulfide bonds are necessary for ST<sub>a</sub> action [11,64,74], binding may involve one or more of the ST<sub>a</sub> disulfide bonds.

Further studies indicated that the ST<sub>a</sub> receptor consists of three peptides (with a combined molecular weight of approximately 200 000) with binding domains for ST<sub>a</sub> [39]. The receptor is not identical with guanylate cyclase since Waldman et al. [85] demonstrated that the ST<sub>a</sub> receptor from membranes of rat mucosa migrated as a 200 000 molecular weight protein; the particulate guanylate cyclase was a 300 000 molecular weight protein. Additionally, Waldman et al. [85] found that thiol reducing compounds (cystamine or *N*-ethylmaleimide) inhibited ST<sub>a</sub> activation of guanylate cyclase but did not inhibit binding of ST<sub>a</sub> to the receptor. Thus, ST<sub>a</sub> receptor and guanylate cyclase behave as independent proteins. Waldman and his coworkers [85] suggested that the two proteins – receptor and guanylate cyclase – are tightly coupled by cytoskeletal components present in the rat intestinal mucosa membrane. The precise nature of the molecular events which lead to activation of guanylate cyclase by the ST<sub>a</sub>-receptor complex has not yet been elucidated.

The genes coding for the expression of the enterotoxins, LT and ST, in ETEC are located on plasmids – the Ent plasmids [68,84]. The Ent plasmids are not usually conjugative but they are mobilized by resident conjugative plasmids. Thus, the Ent plasmids can be readily transmitted throughout the bacterial population. The Ent plasmids have been divided into: ST<sub>a</sub> plasmids, ST<sub>b</sub> plasmids, LT plasmids, ST<sub>a</sub>-LT plasmids, and the ST<sub>b</sub>-LT plasmids. Smith et al. [68] stated that no ST<sub>a</sub>-ST<sub>b</sub> plasmid has been found; more recently however, Echeverria et al. [14] reported an ETEC isolate that contained an Ent plasmid that coded for both ST<sub>a</sub> and ST<sub>b</sub>. The Ent plasmids range in size from 20 to 110 MDa [14,68]. Other virulence properties such as antibiotic resistance [14] and colonization factor antigens [13,78] may be encoded on the Ent plasmids in addition to enterotoxin.

In certain strains of ETEC, the Ent genes are located on transposable elements (transposons). So et al. [70] and So and McCarthy [71] demonstrated that the plasmid-borne ST<sub>a</sub>-coding region from a bovine ETEC strain was located within a transposable DNA element. Similarly, the ST<sub>b</sub> gene in some strains of ETEC has been found to be a part of a transposon [41,46].

In strains of ETEC producing ST<sub>a</sub>, the synthesis of toxin was not observed in media containing glucose, gluconate, or L-arabinose. When glycerol or pyruvate were used as carbon sources, decreased levels of ST<sub>a</sub> resulted [2,3]. Addition of c-AMP reversed the glucose repression of ST<sub>a</sub> [3,45]. Thus, synthesis of ST<sub>a</sub> (and presumably ST<sub>b</sub>) is subject to catabolite repression which is reversible by addition of exogenous c-AMP. Sugars that repress ST<sub>a</sub> synthesis support the highest levels of LT production [26]. Obviously, the synthesis of ST<sub>a</sub> and LT have different regulatory mechanisms.

#### ST<sub>b</sub>

ST<sub>b</sub> – the methanol-insoluble heat-stable enterotoxin elaborated by ETEC strains – has not been studied in such great detail as ST<sub>a</sub>. Part of the reason lies in the cumbersome assay: ST<sub>b</sub> is detectable only in ligated intestinal segments of weaned pigs. It is inactive in suckling mice, as well as in rat and rabbit ligated intestinal loops [34]. Oral challenge of piglets with ETEC strains that produce only ST<sub>b</sub> enterotoxin did not lead to diarrhea even though the toxin isolated from these strains was positive in pig ileal loop assays [48]. Another reason for the lack of study is that ST<sub>b</sub> does not cause diarrheal illness in humans, since human ETEC strains do not appear to produce ST<sub>b</sub> [87]. ST<sub>b</sub> does not elevate levels of c-GMP (or c-AMP) in intestinal mucosa [34,86] and appears to initiate a secretory response in intestinal mucosa by a mechanism independent of cyclic nucleotides. Nothing is known about ST<sub>b</sub> receptor sites in intestinal tissue.

The nucleotide sequence of the ST<sub>b</sub> gene is quite different from that of the ST<sub>a</sub> genes [42], thus the two heat-stable enterotoxins are genetically distinct and there is no amino acid homology between the two toxins. According to Lee et al. [42], ST<sub>b</sub> con-

tains 49 amino acid residues with a precursor peptide containing 71 amino acids. It is rich in cysteine residues which are arranged differently than in ST<sub>a</sub>. Picken et al. [57] stated that ST<sub>b</sub> contained 48 amino acids including four cysteine residues distributed evenly throughout the toxin molecule. The precursor molecule consisted of 71 amino acid residues.

When the ST<sub>b</sub> gene was cloned in mini- or maxi-cells, Spandau and Lee [72] could not detect toxin. The failure to isolate toxin appeared to be due to the low level of expression of the gene. The ST<sub>b</sub> gene is regulated by a weak promoter which is capable of binding RNA polymerase but may be a poor initiator of transcription, and therefore only small amounts of ST<sub>b</sub> are produced [72].

ST<sub>b</sub> is not cell-bound but instead is released directly into the medium [42]. LT is cell-associated and is localized in the periplasmic space [30] whereas ST<sub>a</sub>, similarly to ST<sub>b</sub>, is transported and released into the exterior medium [31]. It is obvious that more studies on ST<sub>b</sub> are needed in order to understand its genetics and regulation, mechanism of action and its role, if any, in disease.

#### STs PRODUCED BY OTHER MICROBIAL SPECIES

An enterotoxin similar to ETEC-ST<sub>a</sub> is produced by certain strains of *Yersinia enterocolitica*. The toxin is positive in the suckling mouse and ileal loop assays and is heat-stable at 121°C for 30 min [7,56]. Unlike ST<sub>a</sub>, *Yersinia* ST (Y-ST) is not formed when the organism is grown at 37°C but is found only when *Y. enterocolitica* is grown at temperatures below 30°C; 26°C appeared to be optimum [56]. Even though *Y. enterocolitica* grows reasonably well at 4°C, Y-ST is not produced at that temperature [18]. Similarly to ETEC-ST<sub>a</sub>, Y-ST synthesis is repressed in glucose-containing media [7].

The mode of action of Y-ST is similar to that of ETEC-ST<sub>a</sub>: fluid accumulation in intestinal mucosa due to the stimulation of guanylate cyclase with concomitant increase in c-GMP levels [59,61]. A tissue culture model indicated that Y-ST increased the levels of c-GMP whereas ETEC-ST<sub>a</sub> had no effect [32].

Y-ST contains 30 amino acid residues and there is a great deal of similarity to ETEC-ST<sub>a</sub>, in particular with regard to the cysteine residues [79]. Antisera against Y-ST neutralize the biological action of ETEC-ST<sub>a</sub> and vice versa but the heterologous reactions are weaker than the homologous reactions, indicating that the two STs have common antigens as well as unique characteristics [55]. Inoue et al. [33] synthesized the 30-amino-acid peptide corresponding to the known structure of Y-ST as well as a shorter amino acid portion (including amino acids 15 through 30) of Y-ST and found that the biological activity of the two peptides was very similar to that of Y-ST isolated and purified from culture media.

The role of Y-ST in disease is questioned because toxin is not formed at 37°C [18,56,65]. Schiemann [65] has shown that *Y. enterocolitica* serotype 0:3 strains that were positive for invasiveness and autoagglutination at 35°C produced diarrhea in mice whether or not they produced toxin. An 0:3 strain that was invasive and produced Y-ST but was negative for autoagglutination did not produce diarrhea. Virulent strains of *Y. enterocolitica* gave a positive Sereney reaction in guinea pig cornea, were lethal for mice i.p. (LD<sub>50</sub>, approximately 10<sup>2</sup> cells) and p.o., and produced Y-ST [23]. Loss of a 42-MDa plasmid led to loss of these virulence properties but did not eliminate production of Y-ST. Thus, the work of Gemski et al. [23] and Schiemann [65] indicate that Y-ST production is not related to diarrhea in mice nor related to virulence in *Y. enterocolitica*.

In summary, Y-ST appears to be related chemically, immunologically, and in mode of action to ETEC-ST<sub>a</sub>. The role of Y-ST in diarrhea is questioned because it is not elaborated at 37°C and non-toxigenic strains of *Y. enterocolitica* can produce diarrhea. However, toxin preformed in foods stored at temperature-abuse conditions (20–30°C) could possibly cause disease if the food is consumed [77]; that possibility does not appear to have been explored.

Klipstein et al. [35] demonstrated that *Klebsiella pneumoniae* produced an ST (K-ST) which is active in the suckling mouse assay. Purified K-ST was as

potent as purified ETEC-ST<sub>a</sub> on a weight basis. K-ST was stable at 100°C for 30 min and was not affected by trypsin. Treatment of the toxin with reducing agents abolished its secretory activity in the suckling mouse [35]. Immunologically, K-ST cross-reacted with ETEC-ST<sub>a</sub>, indicating that they are closely related.

ST purified from *Vibrio cholerae* non-01 strains (NAG-ST) did not cross-react with cholera antitoxin but immunological cross-reaction did occur between NAG-ST and ETEC-ST<sub>a</sub> [5]. The toxin was heat-stable (100°C for 30 min), active in suckling mouse assay and was inactivated by reducing agents. NAG-ST contained 17 amino acids, six of which were cysteine [5]. Therefore, the data obtained with NAG-ST indicated that it is similar to but not identical with ETEC-ST<sub>a</sub>.

Strains of *Citrobacter freundii* isolated from Italian children suffering from diarrhea were shown to produce a heat-stable enterotoxin which was active in the suckling mouse assay [27]. The molecular weight of the *Citrobacter* ST was estimated to be 2000–10 000 Da; the toxin was methanol-soluble, stable to acid but not stable above pH 8, and stable at 100°C for at least 10 min. Biological activity was lost on treatment with 2-mercaptoethanol. Thus, *Citrobacter* ST behaves similarly to ETEC ST<sub>a</sub>. In addition, Guarino et al. [27] showed that *Citrobacter* ST cross-reacted with monoclonal antibodies against ETEC-ST<sub>a</sub>. Interestingly, no LT-producing *C. freundii* strains were found.

A virulence plasmid coding for colonization factor antigen I and ST<sub>a</sub> found in an ETEC strain was transferred into and was maintained stably in other members of the family Enterobacteriaceae [88,89]. These included species of *Shigella*, *Salmonella*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Edwardsiella*, *Serratia*, and *Proteus*. *Y. enterocolitica* and *Hafnia alvei* failed to incorporate the virulence plasmid. Some of the strains that received the plasmid were tested in the suckling mouse and rabbit ileal loop assays and were found to increase intestinal fluid accumulation [88]; therefore, the gene was expressed in the recipients. Host bacteria not receiving the virulence plasmid were negative in animal assays.

STs can, therefore, be found in a number of microbial species other than ETEC and it would appear that the ETEC virulence plasmid encoding ST<sub>a</sub> expression can be transferred to a large number of genera within the family Enterobacteriaceae. There is no reason why the carriers of the newly acquired trait could not become widely distributed in nature and thus have the capacity to become important food- and waterborne pathogens.

#### ISOLATION OF ST-PRODUCING ETEC STRAINS FROM FOODS

Enterotoxigenic *E. coli* have been found in a variety of foods and have been implicated in several outbreaks of foodborne illness. Kornacki and Marth [38] have reviewed various aspects of foodborne enteropathogenic *E. coli* including the ETEC and discussed the various types of foods implicated in outbreaks. Mehlman and Romero [47] have proposed procedures and media for the isolation of enteropathogenic *E. coli* from foods. Their procedures involve both a solid-repair system (for food samples with more than 10 enteropathogenic *E. coli*/g food) and a liquid-repair enrichment system (for samples with less than 10 cells/g). A repair system is necessary because of the possibility that the food sample contains sublethally stressed (injured) cells which will not grow on most selective media [69]. In the solid-repair procedure [47], dilutions of the foods are spread onto cellulose acetate membranes which are then transferred to the surface of BHI agar plates for 3 h at 35°C to effect repair of any injured cells. Rowley et al. [63] cautioned that the length of the repair period depends on the type of injury. The 3 h repair period suggested here may not be appropriate for all food samples and a longer period of repair may be necessary. After repair has taken place, the membranes are transferred to the surface of a selective agar and incubated at 44°C. Suspect *E. coli* colonies are tested for ST production.

In the liquid-repair system [47], the food sample is added to BHI broth and incubated at 35°C for 3 h to effect repair of injured cells. Again, the time period for repair may have to be extended depend-

ing on the type of injury that the bacterial cells may have incurred. After repair, the BHI fluid is transferred to double-strength tryptone phosphate broth and incubated at 44°C for 20 h. The culture is then streaked onto McConkey agar and incubated at 35°C. Suspect *E. coli* colonies are tested for ST production.

The repair, growth and ST<sub>a</sub> production by ETEC after copper-induced injury has been studied by Singh and McFeters [67]. Copper-injured ETEC strains were sensitive to 0.1% deoxycholate and repair (regaining of tolerance for deoxycholate) was effected in BHI medium or in a defined amino acid medium. ST production by injured-repaired ETEC lagged behind that of uninjured cells; upon extended incubation, however, the injured-repaired ETEC strains did produce ST levels comparable to that of uninjured controls [67]. The effect of sublethal heat or other forms of sublethal stress on the stability of the ST plasmid and on production of ST in ETEC has not been studied.

Hill and Carlisle [28] pointed out that enrichment for ETEC utilizing sodium lauryl sulfate-containing media incubated at 44.5°C can lead to loss of virulence plasmids with concomitant failure to detect ETEC strains that produce enterotoxins. The conditions and media used for transport and storage of ETEC strains may affect the detections of ST<sub>a</sub>-producing organisms since the loss of the virulence plasmids may be facilitated under certain conditions of temperature, storage time, and type of medium [83]. Obviously, more studies are needed to improve the media and isolation conditions for ETEC to ensure the maximum recovery of organisms with intact virulence plasmids.

#### DETECTION AND QUANTITATION OF ST<sub>a</sub>

##### *Bioassay*

The suckling mouse assay, described by Giannella [24], is specific for ST<sub>a</sub>; no fluid accumulation in suckling mice is noted with LT, ST<sub>b</sub>, or cholera toxin. A minimum of 0.4–2.6 ng of ST<sub>a</sub> (depending on the purity of the preparation) is required to give a positive reaction [25]. While quite specific, the

suckling mouse assay is only semiquantitative and is cumbersome to perform. It is not suited for routine assays involving large numbers of ETEC isolates.

#### *Radioimmunoassay (RIA)*

By conjugating purified ST<sub>a</sub> to bovine serum albumin or bovine immunoglobulin, Giannella et al. [25] and Frantz and Robertson [21] produced anti-ST<sub>a</sub> which was used in RIA. The RIA measured ST<sub>a</sub> quantitatively and reproducibly in the range of 50–500 pg and the assay could be used with complex bacteriological media. Thus, no extensive work-up of the sample was necessary to do the assay. The RIA was specific for ST<sub>a</sub>; there was no cross-reaction with LT, ST<sub>b</sub>, staphylococcal enterotoxin B, or cholera toxin. ST<sub>a</sub>s from bovine, porcine, or human ETEC strains reacted similarly. There was excellent correlation between the suckling mouse assay and RIA [21,25]. While the RIA is quite sensitive, the use of <sup>125</sup>I-labeled antigen requires highly trained personnel, sophisticated and expensive equipment and disposal of radioactive materials.

#### *Enzyme-linked immunosorbent assay (ELISA)*

A number of workers have produced either polyclonal or monoclonal antibodies which were used to develop ELISAs capable of detecting and quantitating ST<sub>a</sub>s of human or porcine ETEC origin when human ST<sub>a</sub> was conjugated to porcine immunoglobulin G, bovine serum albumin or human thyroglobulin. Klipstein et al. [37] found that the minimum detectable amount of ST<sub>a</sub> in direct double-sandwich ELISA was 140 pg/ml of bacterial culture fluid, versus a minimum of 40 ng/ml for the suckling mouse assay. Thompson et al. [81,83], and Ronnberg et al. [62] developed competitive ELISAs for ST<sub>a</sub>; Thompson et al. employed monoclonal antibodies whereas Ronnberg et al. used polyclonal antibody. Depending on the monoclonal antibody used, as little as 100 pg of ST<sub>a</sub> could be detected. The monoclonal antibodies did not react with LT, cholera toxin, staphylococcal enterotoxin B or ST<sub>b</sub>. There was complete agreement between the suckling mouse assay, RIA, and monoclonal ELISA for ST<sub>a</sub> [83].

Svennerholm et al. [75,76] have developed a competitive ST<sub>a</sub>-GM1-ELISA for detection of stable toxin. The ELISA is based on the competitive inhibition by free ST<sub>a</sub> of ST<sub>a</sub> antibody binding to GM1-ST<sub>a</sub>-cholera subunit B conjugate; both polyclonal and monoclonal antibody systems were used. The ST<sub>a</sub>-GM1-ELISA was sensitive enough to detect 50–200 pg ST<sub>a</sub>.

The ELISAs described above were suitable for the quantitation of ST<sub>a</sub> as well as for screening large numbers of ETEC isolates.

#### *DNA diagnostic techniques*

A number of <sup>32</sup>P-labeled DNA probes suitable for colony hybridization studies have been developed for the detection of ST<sub>a</sub>-producing strains. DNA probes are labeled specific DNA fragments from ST<sub>a</sub> genes which are used to probe for homologous DNA in ETEC strains under study.

Moseley et al. [52], using a DNA probe isolated from the ST<sub>a</sub> gene present in a porcine strain of ETEC, found that not all ST<sub>a</sub>-producing strains from human sources (and positive in the suckling mouse assay) had genetic homology with the probe. Therefore, the authors concluded that there must be more than one gene for ST<sub>a</sub> production. Use of two different ST DNA probes, one isolated from a porcine strain of ETEC (ST<sub>a</sub>-P; ST-Ia) and the other of human origin (ST<sub>a</sub>-H; ST-Ib), allowed the detection of all ST<sub>a</sub>-producing strains that were positive in the suckling mouse assay [50,51]. The gene for ST<sub>a</sub>-H codes for the production of the 19-amino-acid ST and the ST<sub>a</sub>-P gene codes for the 18-amino-acid toxin. The colony hybridization DNA probes developed by Moseley et al. [50,51] allowed detection of ETEC strains inoculated directly onto nitrocellulose filters or from stool samples spotted onto filters. Toxin producers could be detected in water samples containing more than 10 ETEC/ml and the probes could detect ST<sub>a</sub>-positive ETEC in stool samples containing one ETEC/100–1000 cells of normal intestinal flora [51].

By using three ST<sub>a</sub> gene probes which they termed ST-Ia, ST-Ib, and ST-Ic, Maas and co-workers [43] were able to identify all suckling mouse-positive ST<sub>a</sub>s from ETEC strains isolated in

Brazil. The ST-Ib and ST-Ic probes were similar, with 93% homology, whereas the ST-Ia probe had only 70% homology with the other two probes. Only about 50% of the ETEC strains (positive in the suckling mouse assay) isolated from necropsied calves hybridized with an ST<sub>a</sub>-P DNA probe and none reacted with an ST<sub>a</sub>-H probe [44] even though the ST<sub>a</sub>-H probe did react with strains known to produce ST<sub>a</sub>-H toxin. Thus, there must be a gene(s) encoding for heat-stable suckling mouse-positive enterotoxin(s) which is at present unknown and different from ST<sub>a</sub>-H.

Hill et al. [29], Echeverria et al. [15,16] and Murray et al. [54] prepared synthetic single-stranded <sup>32</sup>P-labeled oligodeoxyribonucleotide probes for detecting the ST<sub>a</sub>-H and ST<sub>a</sub>-P genes in ETEC strains. ST<sub>a</sub>-producing ETEC colonies could be detected by colony hybridization even when there was a large excess of nontoxicogenic *E. coli* cells present. There were no positive samples by biological assay that were negative by probe assay. Echeverria et al. [15,16] and Murray et al. [54] recommended the use of synthetic DNA probes for detection of ST producers since gene probes made from cloned DNA fragments may give nonspecific results if they contain plasmid vector DNA.

In the suckling mouse assay, RIA, or ELISA, ST<sub>a</sub> from different sources behave similarly. DNA hybridization is considerably more sensitive since it can detect different genes which appear to produce very similar stable toxins. DNA colony hybridization techniques are particularly useful in screening for ST<sub>a</sub> producers in large numbers of ETEC isolates in comparison to the lengthy procedures involved in testing individual *E. coli* colonies for ST<sub>a</sub> production. Colony hybridization is invaluable for assaying colonies from stool samples since pure cultures are not necessary for hybridization. However, the use of <sup>32</sup>P as the DNA label limits the use of colony hybridization because of the short half-life of <sup>32</sup>P as well as the inconvenience inherent in the use of radioisotopes.

Other methods for labeling DNA are available which are not as sensitive as radioisotope labeling but should make colony hybridization suitable for use in non-research areas. In addition to <sup>32</sup>P-label-

ing, Mulcahy [53] suggested labeling DNA with biotin. The biotin-labeled probe is allowed to hybridize to complementary DNA sequences and then avidin conjugated to an enzyme is added. Detection is accomplished by adding a substrate that gives a colored product when attacked by the avidin-enzyme-DNA-biotin complex. Biotin-labeled probes have a long shelf life and require no special precautions in their use. Another procedure is labeling the probe DNA with an enzyme. Detection of hybridization is accomplished by using an enzyme substrate that produces a colored product [53]. Using lysates of ETEC and biotin-labeled DNA probes, Bailkowska-Hobrzanska [6] developed a dot blot hybridization test for detection of ST<sub>a</sub>-H and ST<sub>a</sub>-P producers. The results obtained with the biotinylated DNA probes agreed well with those obtained in both the suckling mouse assay and <sup>32</sup>P-labeled DNA ST<sub>a</sub> probes. The biotinylated probes were found to be five- to 10-fold less sensitive than the corresponding <sup>32</sup>P-labeled probes [6]. Echeverria et al. [16] point out that biotinylated probes are as sensitive and specific as <sup>32</sup>P-labeled DNA probes for detection of enterotoxin genes in cell lysates but the biotin-labeled probes are not sensitive enough to use on colonies or stool samples fixed on nitrocellulose or Whatman 541 filters.

#### FUTURE RESEARCH NEEDS

Of vital concern to food microbiologists is the lack of selective media for *E. coli* which ensure the retention of the enterotoxigenic character, i.e., the virulence plasmid of ETEC strains. Loss of the virulence plasmid due to the action of inhibitory media components leads to failure to detect ETEC strains. A selective medium also should allow injured ETEC strains to repair and subsequently form colonies. Ideally, the medium should be selective enough to allow only ETEC to grow, but this is probably impossible to achieve. DNA hybridization of individual *E. coli* colonies on the selective agar will allow the food microbiologist to determine the number of ETEC cells that were present in the original population. <sup>32</sup>P-labeled DNA probes are

not practical for use in field, clinical or industrial food microbiology laboratories. Biotinylated DNA probes do not have the difficulties inherent in the use of  $^{32}\text{P}$ -probes but they lack the sensitivity of the radiolabeled probes. Development of nonradioactive DNA probes for ETEC strains with the sensitivity of  $^{32}\text{P}$ -labeled DNA probes should be actively pursued.

More knowledge is needed concerning the nature of the receptor sites for  $\text{ST}_a$  and how the receptor- $\text{ST}_a$  complex activates guanylate cyclase. Research in this area should provide information on how to alleviate or prevent the diarrheal syndrome and perhaps even provide information on how to protect young children from potentially fatal diarrhea.

Heat-stable and suckling mouse-positive toxins are produced in various species of the family Enterobacteriaceae other than *E. coli*. Since many of these organisms are or have the potential of being food poisoning bacteria, more information is needed to determine the role of these STs in diarrhea.

Finally, it is important to determine whether  $\text{ST}_b$  has a role in diarrhea in animals and man. Rapid assay methods are needed for the detection and quantitation of  $\text{ST}_b$ . The weaned pig ileal loop assay is too cumbersome to use as a method for gaining much-needed information about  $\text{ST}_b$ . Until a better and faster assay is developed, research on  $\text{ST}_b$  will not advance very far.

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