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DETECTION OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI* 0157:H7 BY RADIOACTIVE AND NONRADIOACTIVE DNA PROBES

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Yen-Ping Kuo

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Douglas W. Eby
Major professor

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DETECTION OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI* O157:H7 BY RADIOACTIVE AND NONRADIOACTIVE DNA PROBES

By

Yen-Ping Kuo

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ABSTRACT

DETECTION OF ENTEROHEMORRHAGIC *ESCHERICHIA COLI* O157:H7 BY RADIOACTIVE AND NONRADIOACTIVE DNA PROBES

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Enterohemorrhagic *Escherichia coli* serotype O157:H7, associated with hemorrhagic colitis and hemolytic-uremic syndrome, has now emerged as an important enteric pathogen. Shiga-like toxin synthesis has been strongly implicated as a factor contributing to the pathogenesis of EHEC. The DNA probes derived from the genes for SLT were used for identifying EHEC with a radioactive and three nonradioactive DNA detection systems on bacterial colony blots. Detecting SLT-production in seventy-four bacteria strains with these probes and DNA detection systems (^{32}P-DNA, BlueGENE™, Genius™, Chemiprobe™) provided 100% specificity and sensitivity compared to the CDC Verotoxin assay. The DNA probes detected *E. coli* O157:H7 with 100% accuracy. Some of the hybridization parameters that influenced the specificity and sensitivity of the three nonradioactive DNA detection systems used were also evaluated.

This is dedicated to my husband Tenfu Chang
and
our lovely daughter Yi-Shin Chang.

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RESEARCH PROBLEM

Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7, initially recognized in 1982 in the United States, has now emerged as an important enteric pathogen of considerable public health significance in the United States, Canada, and the United kingdom. Many outbreaks and numerous sporadic cases of hemorrhagic colitis, hemolytic-uremic syndrome and diarrheal illness have been reported. These are associated with high morbidity and mortality. In view of the importance of *E. coli* serotype O157:H7 in human diseases, identification of the organism is extremely important.

It has been reported that EHEC synthesizes elevated levels of Shiga-like cytotoxin. Biological identification of these toxins are usually accomplished by antibody-dependent assays, which are inconvenient to use; especially when screening large numbers of isolates. Nucleic acid hybridization has been previously demonstrated as a reliable technique which can facilitate detection of bacteria containing similar pathogenic characteristics. The major obstacle to wider application of nucleic acid hybridization in diagnostic microbiology is the fact that current techniques routinely use nucleic acid probes labeled with radioisotopes. The development of nonradioactive hybridization protocols that are adequate for particular applications is highly desirable.

LITERATURE REVIEW

Characteristics of *Escherichia coli*

Escherichia coli, which belongs to the family Enterobacteriaceae and genus Escherichia, is the currently accepted name for the common coliform bacillus originally called *Bacillus coli commune* by Escherich in 1885, *Bacillus coli* by Migula in 1895, and *Bacterium coli* by Lehmann in 1896 [78]. *E. coli* is a non-sporeforming, gram-negative rod. When grown in broth it produces a well-dispersed turbidity. Most strains have flagella and are motile. Smooth strains form shiny, convex, colorless colonies but when repeatedly subcultured they become rough and form lusterless, granular colonies. Encapsulated variants produce mucoid colonies, particularly when incubated at low temperatures and when grown in media low in nitrogen and phosphorus but high in carbohydrate. Typical *E. coli* colonies are usually easy to recognize by their characteristic appearance on certain differetial media. They are lactose fermenters, and on eosin-methylene blue and Endo agars they have a metallic sheen. However, some strains ferment lactose late, irregularly, or not at all. On blood agar some strains produce beta-hemolysis, though most strains are non-hemolytic [40,78,158]. For direct isolation, the less inhibitory media, such as MacConkey agar and EMB agar, are recommended. Blood agar plates should also be used, because certain enteropathogenic strains may not grow on MacConkey agar, but will grow well on blood plates [40]. Pure cultures of *E. coli* will give the reactions shown in table 1 [40,78,158].

In the 1940s, Kauffman [73] proposed a scheme to differentiate *E. coli*

on the basis of lipopolysaccharide O, flageller H, and polysaccharide K antigens. Together, these constitute the O:H system, which has played an important role in studies of the epidemiology and pathogenesis of *E. coli* infection.

Table 1. Biochemical reactions of *Escherichia coli*

Oxidase test	-
Indole	+
Methyl red	+
Voges-proskauer	-
Simmons' citrate	-
Hydrogen sulfide (TSI)	-
Urease	-
KCN	-
Motility	variable
Gelatin (22° C)	-
Lysine decarboxylase	d
Arginine dihydrolase	d
Ornithine decarboxylase	d
Phenylalaniline deaminase	-
Malonate	-
Gas from glucose	+
Lactose	+
Sucrose	d
Mannitol	+
Dulcitol	d
Salicin	d
Adonitol	-
Inositol	-
Sorbitol	d
Arabinose	+
Raffinose	d
Rhamnose	d

d: May be delayed.

Role of *Escherichia coli* in Nondiarrheal Human Disease

As the predominant species among the facultative anaerobic normal flora of the intestine, *Escherichia coli* plays an important role in maintaining intestinal physiology [32]. Most strains are nonpathogenic in the bowel. Within this species, however, there are fully pathogenic strains that are associated with a number of disease syndromes.

E. coli most commonly causes disease in the urinary tract. It has been hypothesized that the major reservoir for uropathogenic *E. coli* in humans is the large intestine [173] and that the colonization of the vaginal introitus via fecal contamination may precede infection of the bladder (cystitis) [43]. It is now clear that at least some uropathogenic *E. coli* strains express gene products that appear to be directly involved in the colonization of the urinary tract. These gene products, which include an adhesin (P adhesin) and a pilus, are encoded by the pyelonephritis-associated gene (*pap*) cluster [118,119].

E. coli is often found, along with other enteric bacteria, in sepsis adjacent to the gut, such as peritonitis, appendicitis, and infections of the gallbladder and biliary tract. It occurs on the skin of the perineum and genitalia and frequently infects wounds that become contaminated with urine or feces [90,158]. *E. coli* can also cause meningitis and is now the most encountered species in gram-negative septicemia, resulting in severe shock resembling that produced by intravenous injections of endotoxin in laboratory animals [158].

Diarrheagenic *Escherichia coli*

E. coli is a well-known opportunistic pathogen when outside its normal ecological niche. Although *E. coli* has been suspected for many years as a

possible etiologic agent of diarrheal diseases within its habitat, it was not established until the mid-1940s [17,165]. Further detailed studies of the pathogenic mechanisms involved permit the separation of diarrheagenic *E. coli* into five major categories [86] (1) Enterotoxigenic *E. coli* (ETEC) that adhere to the mucosa of the small bowel and produce a heat-labile (LT) toxin, a heat-stable (ST) toxin, or both; (2) Enteropathogenic *E. coli* (EPEC) that adhere to the mucosa of the small intestine and produce a characteristic effacement of microvilli; (3) Enteroinvasive *E. coli* (EIEC) that produce neither LT nor ST, but like *Shigella*, penetrate and multiply within the epithelial cells lining the colon; (4) Enterohemorrhagic *E. coli* (EHEC), this group causes hemorrhagic colitis and hemolytic uremic syndrome and are of serotype O157:H7; and (5) Enteroadherent *E. coli* (EAEC), that is less-well-defined and so far identifiable only by their pattern of adherence to HEp-2 cells in tissue culture. These categories are based on distinct virulence properties, different interactions with the intestinal mucosa, distinct clinical syndromes, differences in epidemiology, and distinct O:H serotypes [table 2].

ETEC Enterotoxigenic *Escherichia coli* recognized by De et al. in 1956 [28] can produce either or both a heat-stable and a heat-labile enterotoxin, depending on the plasmid(s) they carry. ETEC are assuming a major role in diarrheal diseases, particularly in the developing world. They are now known to be [86,144]: (1) one of the common causes of infant diarrhea in less-developed countries [12]; (2) the agent most frequently responsible for travelers' diarrhea [108]; (3) a common cause of the cholera syndrome in native adults living in cholera-endemic areas [107]; (4) an occasional source of outbreaks of diarrhea [107]; (5) an infection correlated with adverse nutritional consequences [12].

Table 2. O Serogroups associated with the major categories of diarrheagenic *E. coli*[a].

Classical enteropathogenic *E. coli*
* Most important (Class I, usually EAF+)
O55, O86, O111, O119, O125, O126, O127, O128ab, O142.
* Less important (Class II, rarely EAF+)
O18, O44, O112, O114.
Enterotoxigenic *E. coli*
O6, O8, O15, O20, O25, O27, O63, O78, O80, O85, O115, O128ac, O139, O148, O153, O159, O167.
Entetoinvasive *E. coli*
O28ac, O29, O124, O136, O143, O144, O152, O164, O167.
Enterohemorrhagic *E. coli*
O157, O26, O111.
Enteroadherent *E. coli*
O serotypes not yet defined.

a: Law, 1988 [82]; Levine, 1987 [86].

ETEC possess plasmid-mediated attachment or colonizational factors (CFAI and CFAII) [27,86,125,144] which have been shown to aid in colonization of the small bowel and subsequent production of disease by allowing the bacteria to overcome the peristaltic defense mechanism of the small intestine [86,144]. Accumulated evidence suggests that ETEC strains isolated from patients with diarrhea commonly belong to a small number of serogroups or serotypes [44,125]. Usually, these serotypes elaborate both LT and ST and possess fimbrial colonization factors [125,126]. By molecular analysis [27], it has been shown that serotypes of ETEC strains associated with diarrhea (classical strains) such as 06, 025, 027, 0128, and 0159 resulted from the dissemination of ancestral clones which received enterotoxin plasmids long ago.

ETEC infection is acquired by ingesting contaminated food or water. The clinical features of ETEC infection are watery diarrhea, nausea, abdominal cramps, and low-grade fever [86]. The definitive identification of strains of ETEC is the demonstration that they produce enterotoxins, since they have no colonial or biochemical characteristics that distinguish them from other strains of *E. coli* [86,144].

EIEC Enteroinvasive *Escherichia coli* comprises those *E. coli* strains that possess a high-molecular-weight (about 140 MDa) virulence plasmid closely related to the virulence plasmid of *Shigella* [56]. These strains, described by DuPont et al.[34] in 1971, resemble *Shigella* in many ways. Like *Shigella*, their cardinal pathogenic feature is the capacity to invade and proliferate within epithelial cells resulting in eventual death of the cells [34]. The invasive capacity of both EIEC and *Shigella* is dependent on the presence of large plasmids [56] coding for the production of several outer membrane proteins involved in invasiveness [52]. But, none of the known EIEC serogroups from several geographic regions was found to produce Shiga-like cytotoxic activity when assayed in a Hela cell system [23]. EIEC often resembles *Shigella* in being nonmotile and unable to ferment lactose. Furthermore, EIEC and *Shigella* O antigens show many cross-reactions [88].

EIEC infection, clinically, is marked by fever, severe abdominal cramps, malaise, toxemia, and watery diarrhea followed by gross dysentery consisting of scanty stools of blood and mucus [86]. EIEC can be diagnosed by serotyping suspect *E. coli* strains [168], by an ELISA that detects the outer membrane proteins associated with invasiveness [129], and by DNA probes that detect the gene for invasiveness [181].

EPEC Enteropathogenic *Escherichia coli* represent the first recognized

diarrheagenic class of *E. coli*, having come to light in the 1940s and 1950s as the cause of epidemic and sporadic infant diarrhea [82,88,89]. The classic EPEC strains have been found to lack the properties of heat-labile and heat-stable enterotoxin production and enteroinvasiveness [48].

EPEC strains cause distinctive ultrastructural histopathologic lesions in human intestines [134]. The distinctive lesions involve destruction of the microvilli by the EPEC bacteria, typically without further evidence of invasion [88]. The majority of strains from the most commonly incriminated O serogroups adhere in localized clusters to HEp-2 cells in tissue culture [6,82,114,115]. This property, which has been labeled "EPEC Adherence Factor (EAF)", is mediated by plasmids 55 to 70 MDa in size [6,82,87,114,115] and is rare in *E. coli* other than EPEC [86]. EAF plasmids encode a 94 kDa outer membrane protein which may be involved in the adherence process [82,87]. EPEC isolates of certain other, less common, O serogroups (044, 086, and 0114) are rarely HEp-2 adhesive. These EPEC, designated class II, possess distinct 50-70 MDa plasmids lacking EAF genes. They have been proven to be pathogenic by a mechanism not involving HEp-2 adhesiveness [87].

Nataro et al. [114] have cloned a 1-kilobase segment of DNA from the EAF plasmid of strain E2348/69, and have shown it to be a highly sensitive and specific DNA probe for detecting EPEC that carries the EAF plasmid. Clinically, EPEC illness is characterized by fever, malaise, vomiting, and diarrhea with prominent amounts of mucus but without gross blood [86].

EAEC Enteroadherent *Escherichia coli* are EAF-negative, nonclassical serotype strains described by Mathewson et al in 1985 as a cause of travelers' diarrhea. [102,103]. In their study, it was shown that at least one strain could cause mild but definite diarrhea without blood or fecal

leukocytes [102]. EAEC do not elaborate LT, ST, or elevated levels of Shiga-like toxin, or invade epithelial cells [86]. But, they do exhibit mannose-resistant adherence to HEp-2 tissue culture cells [102].

Vial et al. [171] have characterized this less-known diarrheagenic *E. coli* category. Thirty-nine of 42 strains, which were negative by tests with DNA probes for EPEC, ETEC, EIEC, and EHEC and by serotype, did not fit these categories, and had a 55-65 MDa plasmid. Transfer of the plasmid also transferred the ability to express smooth lipopolysaccharide, fimbriae, and the aggregative phenotype. This suggests that plasmids appear to be important in encoding or expressing some putative virulence properties of EAEC. Toxin involvement was also proved by inoculating live organisms in rabbits and rats. There is no correlation with any specific O group among EAEC. Nevertheless, the recurrence of certain H types, particularly H33, suggests that common serotypes exit.

EHEC The term enterohemorrhagic *Escherichia coli* refers to strains that have the same clinical, epidemiological, and pathogenic features associated with the prototype EHEC organism *E. coli* 0157:H7 [86]. All of the EHEC strains are verotoxin (Shiga-like toxin)-producing *Escherichia coli* (VTEC) [86]. They are now considered to be the major causes of two syndromes of hitherto unknown cause. These are hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) [69].

All EHEC strains have been found to produce high levels of Shiga-like toxins I and/or II [121,122,123,124]. These are distinct from the classic *E. coli* LT and ST enterotoxins and are characterized by their unique toxic effect on Vero and Hela cells in tissue culture [121,122]. In humans, EHEC are thought to release toxins in the bowel where they are absorbed into the blood. Here the toxins are thought to cause endothelial damage of small blood vessels, leading in turn to local intravascular coagulation and

fibrin deposition in the CNS, gut, and kidney. These events invariably lead to microangiopathic hemolytic anemia, thrombotic thrombocytopenic purpura, acute CNS dysfunction, and bowel ischemia and necrosis [36]. SLT-I, by interfering with protein synthesis, can inhibit absorption of salt and water by enterocytes on the villus tips, an act that probably accounts for the initial watery diarrhea of most EHEC infections [121,122,124].

EHEC infection is characterized by severe crampy abdominal pain, initial watery diarrhea followed by grossly bloody diarrhea, unaccompanied by fecal leukocytes, and little or no fever [140]. Antibiotics have not yet proved effective against EHEC. In institutional settings, the organism can be spread by undercooked hamburger and by unpasteurized milk; person-to-person spread has also been reported. In developing countries, the importance and modes of transmission of EHEC are yet unknown [86].

Enterohemorrhagic *E. coli* 0157:H7

Clinical and Epidemiological Significance *Escherichia coli* serotype 0157:H7 is a newly recognized pathogen that has been epidemiologically linked to outbreaks of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) and has been associated with hundreds of sporadic cases of gastrointestinal illness in the United States, Canada, and Great Britain in the past few years [table 3] [57,64,68,69,128,138,140,142,143,156, 157,160,175]. The genetic evidence also strongly supports the fact that isolates of *E. coli* 0157:H7, obtained from geographically separate outbreaks and sporadic cases, belong to a pathogenic clone [177,178]. Historically, 0157:H7 has been a serotype that was rarely isolated from humans or animals [10]. Since 1982, *E. coli* 0157:H7 strains have been frequently recovered from persons with HC or HUS. In recent surveys,

estimates of the frequency of this serotype range from about 0.08% to 1.9% in diarrheal illness and from about 15 to 36% in bloody diarrhea [47,55].

Table 3: Outbreaks of EHEC O157:H7 Infection in Canada and the United States

Date	Location	No. of cases	No. of Deaths
Feb.-Mar. 1982	Oregon	26	
May -June 1982	Michigan	21	
Nov. 1982	Ontario	31	
May 1983	Labrador	19	
Aug. 1983	Alberta	4	
Mar. 1984	Ontario	7	
Sep. 1984	Nebraska	34	4
Sep.-Oct. 1984	North Carolina	36	
Aug. 1985	Ontario	5	
Sep. 1985	Ontario	73	17
Apr. 1986	Ontario	30	
June 1986	Alberta	8	
June 1986	Ontario	2	
July 1986	British Columbia	20	
Oct.- Nov. 1986	Washington	37	2
Dec. 1986	Ontario	4	
June 1987	Alberta	15	2
July 1987	Ontario	9	
July 1987	Ontario	6	
Aug. 1987	Ontario	9	2

Hemorrhagic colitis [140] is a distinct clinical syndrome that presents typically with abdominal cramps and watery diarrhea followed by a hemorrhagic discharge resembling lower gastrointestinal bleeding. After the association between hemorrhagic colitis and *E. coli* 0157:H7 was clearly established [16,17,64,68,140,142,160], studies [21,24,47,69,72,160] showed a close association between EHEC infection and the classical form

of HUS [33,42], a leading cause of acute renal failure in childhood. Classical HUS presents typically a few days after the onset of an acute diarrheal "prodromal" illness. It has been reported that 38%-61% of 0157:H7 infections result in HUS [21,24,47,69,72,140,142,160], and that 75%-90% of patients with HUS in North American have associated EHEC infections of the bowel [36]. Case fatality rates of 0157:H7 infection range from 0% to 10% nation-wide, with higher fatality rates, 3% to 38%, found in elderly residents of nursing homes [21,36,64,142].

Antibiotic treatment is questionable. The use of antibiotics prior to the onset of symptoms is considered to be a risk factor for acquiring the infection [21], though the organism has been shown uniformly susceptible to antibiotics in vitro [135]. The mechanism might involve the enhancement of toxin production by the bacteria or an alternation of the normal competing bowel flora leading to the overgrowth of EHEC [21]. Calcium channel inhibitors have recently been shown to inhibit the cytotoxic action of Shiga-toxin in vitro [146], but the therapeutic value remins to be investigated.

Characteristics and Laboratory Investigation *E. coli* serotype O157:H7 is different from other *E. coli* not only from certain clinical and epidemiological standpoints but also in some bacteriological and molecular features. Krishnan et al. and Ratnam et al. [21,81,135] have reported a 100% negative reaction for beta-glucuronidase and sorbitol, and a 100% positive reaction for raffinose; all strains otherwise were biochemically typical of *E. coli*. All strains produced readily detectable levels of Shiga-like toxin. None were found to mediate hemagglutination of human group A erythrocytes with or without D-mannose. The majority (about 70%) of the strains showed localized and diffuse adherence to HEp-2 cells and Henle 407 cells. Twenty phage types were recognized, and the great

majority of *E. coli* O157:H7 strains belong to phage types 1,2,4, and 8. Plasmid analysis indicated three basic plasmid profiles. Profile I was characterized by 68.7- and 4.2-MDa plasmids. Profile II was characterized by 66.2- and 1.8 MDa plasmids, and profile III was characterized by a 62.5-MDa plasmid. *E. coli* O157:H7 demonstrate attaching and effacing binding to the caecum and colon of orally infected gnotobiotic piglets, chickens and infant rabbits [44]. Organisms adhere to the surface but do not invade the cytoplasm of human epithelial lines in tissue culture. Karch et al. [66] and Tzipori et al. [170] had different reports for whether the 60 Mda general size plasmid plays a key role in the attachment or not. However, more recent findings [152,153,154] suggest that constitutes of outer membrane, but not lipopolysaccharide and H7 flagella, of *E. coli* O157:H7 mediate binding of the organisms to epithelial cells in vitro. This might explain why some *E. coli* O157:H7 strains lacking fimbriae encoded by 60 Mda plasmid can still adhere to HEp-2 cells.

The most important diagnostic procedure for O157:H7 infection is the detection of FVT (fecal verotoxin) [72,81,128], which is also the most sensitive and specific method available. In addition, the demonstration of toxin production by isolated colonies or even from mixed cultures is highly specific. Many laboratories that do not have tissue culture facilities presently use a 1% D-sorbitol-MacConkey medium to detect the EHEC O157:H7; a method that has shown [100] a 100% sensitivity, a 85% specificity, and a 86% accuracy. A diagnositic procedure that completely relies on biochemical reactions has been established by Krishnan et al. [81]; a combination of sorbitol fermentation-negative, raffinose fermentation-positive, and beta-glucuronidase reaction-negative occurs only with O157:H7. More recently, Perry et al. [133] have produced a monoclonal antibody against the O157 antigenic determinant and indicated that it is a more specific typing reagent compared to conventional

polyclonal *E. coli* antisera. Serotyping and H7 antiserum-sorbitol fermentation medium is also valuable [38,55,69,100], but confirmation of toxin production is essential for every strain that is isolated. A so-called VT/PECS {VT (SLT) in polymyxin extracts of colony sweeps} procedure established by Karmali et al. [70] is a useful sensitive screening test for ruling out VTEC. Other SLT-specific methods have been described or are at a developmental stage: a DNA hybridization method using SLT-I and SLT-II-specific DNA probes [58,116,179,180], which has been shown to be at least as sensitive as the detection of FVC; a sandwich enzyme-linked immunoabsorbent assay method reported by Kongmuang et al. [79] in Japan; and a colony blot assay with SLT monoclonal antibodies [67].

Shiga-like Toxin Toxins are proteins that damage host cells and are important in the pathogenesis of infection related to the bacteria that elaborate them. Shiga-like toxin, which is the same as early recognized Vero toxin [120], shares a bifunctional structure with cholera toxin, *E. coli* LT, diphtheria toxin, Pseudomonas exotoxin A, and pertussis toxin. The prototype cytotoxin SLT-I was originally identified in strain H30 by Konowalchuk et al. in 1977 [80]. EHEC Shiga-like toxin was first reported in 1983 by O'Brein et al [123]. It is now further understood that *E. coli* O157:H7 makes two kinds of cytotoxins, one of which can be neutralized by anti-shiga like toxin, designated Shiga-like toxin I (SLT-I), and the other named Shiga-like toxin II (SLT-II) [121,122].

Marques et al [101] classified strains according to the level of cell-associated toxin they produced. The categories were as follows: low, 2×10^2 to 6×10^2 CD_{50} per ml of sonic lysate; moderate, 1×10^3 to 1×10^4 CD_{50} per ml of sonic lysate; and high, 1×10^5 to 1×10^8 CD_{50} per ml of sonic lysate. It was found that moderate and high levels of cytotoxin were almost exclusively found in strains isolated from people with diarrhea,

hemorrhagic colitis, or HUS. This suggests that such elevated levels of cytotoxin may play a role in the pathogenesis of these diseases [162]. Strockbine et al. [162] also observed that the moderate levels of cytotoxin produced were mostly SLT-II, whereas the high levels of cytotoxin were either SLT-I or both SLT-I and SLT-II. When both toxins are produced by the same strain, SLT-I predominates in cell lysates and SLT-II is the more active toxin in culture filtrate [162]. These toxins contain a single A subunit responsible for inhibiting protein synthesis through the catalytic inactivation of 60S ribosomal subunits [18] and 5 copies of a B subunit that bind the toxin to receptors on the cell surface [122]. The SLT-I A and B subunits have MWs of 32,200 and 7,700 [20,122] and the SLT-II A and B subunits have MWs of about 32,000 and 10,200±800 [31,127,182].

The phage-specified structural genes for both SLT-I and SLT-II have been cloned and characterized with respect to nucleotide and amino acid sequences [20,58,63,117,148,162,174,177,178,180]. The structural genes of the two toxins share 58% overall nucleotide and 56% projected amino acid sequence homologies [6]. Both toxins have a similar subunit structure [122], bind to the same glycolipid receptor, Gb3 [174], and inhibit protein synthesis by the same mechanism as Shiga toxin [61,137]. However, they fail to cross-neutralize and show differences in biological activities in tissue culture and animal models [122]. SLT production has also been detected in strains of *Vibrio cholerae* and *Vibrio parahemolyticus* [120]. However, the role this toxin plays in infections due to these bacteria is not yet clear.

Some Molecular Genetic Techniques

During the past several years, the progress in developing the molecular genetic tools for use in the diagnostic laboratory has generated a lot of

excitement among clinical microbiologists. Here, I would like to address some which have been utilized in this study.

Plasmid Isolation Many important bacterial genes are not part of the main chromosome but are part of an autonomous self-replicating extrachromosomal DNA element called a plasmid [26,54,104]. Bacterial plasmids are molecules of double-stranded DNA and are predominantly circular in form [26,54,104]. However, there are some plasmids which have been described in linear form [75]. Plasmids are not essential for normal bacterial growth and their length varies from a few to several hundred kilobase pairs (1 MDa= 1.51 kb) [26,54,104]. They contain genes that are essential for plasmid maintenance functions. Many plasmids contain genes that are useful not only to themselves, but also to their host bacteria. For example, genes controlling drug resistance, degradation of organic compounds, and virulence factors, including the production of toxins [26,54,104] are encoded by plasmids. Most plasmid DNA in bacteria is in the form of a covalently-closed circle (CCC), meaning that there are no breaks in either of the polynucleotide strands which comprise the double-helix. If one of the two strands in a CCC plasmid is broken, or nicked, an open-circle is formed. When both polynucleotide strands are broken a linear molecule is formed [54].

Most of the methods used to isolate plasmids depend on their small size in comparison with the bacterial chromosome, and on their circular form. There are usually three major steps in isolating a plasmid [54,65,93]: 1. Bacteria are broken using a lysis method which does not break CCC plasmid DNA molecules; these methods vary according to the species being investigated. 2. Cell debris and chromosome are removed from the lysate by centrifugation or by an organic solvent. 3. Plasmid DNA is recovered by precipitation. Plasmid DNA can be replicated in either high

or low copy number. The copy number of a high-copy-number plasmid can be increased to several thousand per cell if host protein synthesis is stopped (e.g. by treatment with ampicillin) [25,54].

Isolated plasmids can be further digested with restriction endonucleases [95,130] for estimation of size, for isolation of specific DNA fragments of interest, or for plasmid profile studies [104,109] by agarose gel electrophoresis. Restriction endonucleases are enzymes, isolated chiefly from prokaryotes, that recognize specific sequences within double-stranded DNA. DNA fragments in agarose gel can be recovered by electroelution [46,96,131].

Plasmid Transformation In the laboratory, plasmids can be transferred to bacteria by an artificial process, known as transformation, which was first demonstrated by Mandel & Higa [92]. This method provides a powerful technique which allows a large amount of DNA to be produced. The transformation event can be divided into two general phases: 1) uptake of DNA across the cell envelope; and 2) establishment of that DNA as a stable genetic element in the cells [53,92]. Both gram-positive and gram-negative bacteria can take up and stably establish exogenous DNA. There are some factors to be aware of when using this technique [53]. These include transformation efficiency declines linearly with increasing plasmid size, relaxed and supercoiled plasmids transform with similar probabilities, and each cell is capable of taking up many DNA molecules, so that the establishment of a transformation event is neither helped nor hindered significantly by the presence of multiple plasmids.

DNA probe tests DNA probe tests are now available to the clinical microbiology laboratory for the rapid diagnosis of infectious disease

[15,77,112,113,150]. The DNA probe approach to identification is unique because the focus of the method is the nucleic acid content of the organism rather than the products that the nucleic acid encodes [166]. A DNA probe test is based upon the principle of a nucleic acid hybridization reaction, which can be defined as the formation of stable double-strand nucleic acid molecule from complementary single-strand molecules [37]. A very specific interaction between many complementary base pairs (A-T and G-C) makes DNA probes highly specific in diagnostic tests. The probe test consists of four components [37,94,166]. The first component is the sample or specimen preparation that releases and makes the target nucleic acid available from suspected pathogenic cells, e.g. bacterial colony blot preparations. The second component is the specifically designed DNA probe labeled with either radioactive or nonradioactive reporter molecules. The third component is the hybridization reaction which form the complementary hybrids between probe and target DNA. The formation of specific hybrids is then detected by a suitable detection system.

Southern blotting and bacterial colony blotting: The Southern blotting technique was first established by Southern in 1975 [159] and has been further developed [4,93,167]. The technique involves transfer of electrophoretically separated DNA fragments from an electrophoresis agarose gel onto a nitrocellulose or nylon [136] membrane where DNA fragments are bound and immobilized for convenient hybridization with DNA or RNA. The relative positions of the DNA fragments in the gel are preserved during their transfer to the filter which allows the localization of particular sequences of DNA [4,8,93,159,167]. DNA fragments in the gels are denatured (e.g. by alkaline solution) to single-stranded molecules, neutralized, and transferred to membranes by high salt solution (e.g. 20X SSC). The transferred DNA fragments are then fixed onto the membrane

either by baking or by exposing the membrane to UV light. Acid depurination before denaturation is necessary for improving the transfer efficiency for DNA with larger fragment sizes (≥ 5 kb) [4,8,93,167]. The Bacterial colony blotting technique allows the investigators to screen large numbers of bacterial colonies at the same time. It was first described by Grunstein and Hogness in 1975 [49]. Bacterial colony blotting is accomplished either by transferring bacteria from a master plate to a membrane [49,91,94] or by inoculating bacteria directly onto a sterile membrane overlying on a plate [49,50,94,112,113]. The colonies on the membrane are then lysed and liberated DNA is fixed to the membrane.

DNA labeling: Many different and reliable methods for labeling DNA fragments have been developed over the past few years to generate probes for the detection of DNA and RNA sequences [39,132.139]. In most of the cases, uniformly labeled probes prepared by nick translation [5,132,139] and by random-sequence oligonucleotide-primed synthesis [5,39] are satisfactory. In some cases, when higher specific activities and increased sensitivities are needed, the use of the in vitro synthesis procedures [5,132] turn out to be the best choice. In other cases where intact probes are required, the end-labeling procedure [5,132] should be used. The mechanism of nick translation [5,132,139] involves the combined activities of the 5'→3' polymerase and 5'→3' exonuclease activities of *E. coli* DNA polymerase I. Given a nicked duplex DNA molecule, the polymerase will translocate the nick, removing nucleotides ahead of it using its 5'→3' exonuclease activities, while simultaneously synthesizing DNA at the 3' end. The nicks in the DNA template are generally produced by adding trace amounts of DNase I to the reaction mixture. Random-oligonucleotide-primed synthesis [5,39] is an alternative to nick translation. To carry out the labeling procedure, the DNA molecule

(recovered by ethanol precipitation) is denatured to a linear form by boiling; random-sequence oligonucleotides (typically 6 bases in length) are annealed and then incubate with Klenow fragment [5,95] in the presence of dNTPs. In this way, the hexanucleotides prime the DNA of interest at various positions along the template, and are extended to generate double-stranded DNA that is uniformly labeled on both strands.

Hybridization reactions: After a double-stranded DNA molecule is denatured to single strands, it is capable of reassociating with either a DNA or an RNA strand of complementary sequence [166]. Nucleic acid molecules can tolerate a certain number of mismatched base pairs (e.g. A-C or G-T) and still form stable duplexes. The degree of mismatch that can be tolerated in a hybridization reaction and still maintain a double-stranded molecule is referred to as the "stringency" of hybridization [2,4,94, 161,166]. High stringency [2,4,94,161,166] conditions means that the hybridization reaction is carried out at a high temperature, in low salt concentration, or in high concentration of formamide, which will only allow the annealing of perfectly matched DNA molecules. So, in designing a hybridization experiment, stringency condition must be properly set [2,4,94,161,166]. Hybridization reactions applicable to the clinical laboratory can be performed in four unique formats: on a solid support, in solution, in situ, or by using the southern blot procedure [166]. In a filter hybridization format, the filter is prehybridized with denatured nonspecific DNA, bovine serum albumin and Ficoll [7,94,166]. This solution blocks membrane binding sites and prevents the single-stranded probes in the hybridization solution from nonspecifically sticking to the membrane's surface. The temperature of the washes after hybridization also affects the sensitivity and specificity [2,4,94,161]. The stringency of the washes increases with increasing temperature and decreasing salt concentration.

Detection systems for radioactive and nonradioactive DNA-DNA hybrids: In some cases the reporter molecule is attached or incoporated directly into the nucleic acid backbone of the probe, e.g. ^{32}P-labeled compounds [5,93,94,166]. Considerable effort has been made in recent years to use a second approach for labeling the probes [1,9,13,19,29, 41,45,74,83,84,105,111,166,169,172]. This is an indirect detection system (nonradioactive system), in which the first component is incoporated (or the DNA are chemically modified) into a probe and a second (and even a third) component is added to the reaction mixture after the hybridization has been completed. If the DNA are labeled with radioisotopic compound, after hybridization, the binding of probe DNA to the target DNA can be detected by autoradiography [35,51,59]. This can be done by direct exposure, fluorographic exposure, or exposure with an intensifying screen [35]. The use of intensifying film can shorten the exposure time, or detect lower levels of radioactivity. The radioactive labeling methods are believed to have the highest sensitivity [166]. Unfortunately, radioisotopes are not an attractive alternative for the clinical laboratory. ^{32}P, in particular, has a short half-life (about 14 days), requiring frequent probe preparation. Some nonradioactive labeling and detection systems have been developed through the second approach. Among them the biotin-system, which has the longest history and is now commercially available through BRL ("Bluegene^{TM}") [9], was described initially by Leary et al. [84] and further modified by Chan et al [22]. DNA is incoporated with biotin-dNTP or -NTP and the biotin moiety in the hybrid is then bound with streptavidin conjugated with alkaline phosphatase. After adding a substrate, the colorimetric product is produced. A similar principle is utilized in the digoxigenin-labeled DNA detection system [13] ("Genius^{TM}" system, available through Boehringer Mannheim), but a monoclonal antibody against modified sites is used instead of streptavidin.

Labeling of the cytosine residue with a sulfone group, first described by Budowsky et al. [13,19], is also one of the commercially available nonradioactive DNA detection systems ("Chemiprobe^{TM}" of FMC) [41]. Two monoclonal antibodies are used in this system as a sandwich binding to amplify the signal. The above methods cited were reported to provide satisfactory sensitivity and specificity [11,45,60,76,145,176]. Photobiotin systems, however, have been reported to be slightly less sensitive [176]. Another approach is to attach an enzyme, such as alkaline phosphatase, directly to the DNA by using a 12-atom linker arm [62,141] so that the labeled hybrid can be directly detected by adding substrates. This technique is particular well suited to oligonucleotide probes. There are a variety of alternatives available to radioactive detection systems, some of which may surpass the sensitivity of the isotope.

MATERIAL AND METHODS

Bacterial Strains and Nucleic Acids

E. coli HB101 with recombinant plasmids were kindly provided by J. Newland and R. Neill. In order to allow removal of the probe fragment in a single digestion step, the restriction sites have been duplicated by multiple cloning. The Shiga-like toxin I and II probe genes were first cloned into vector pUC18 , and sequentially, the probe fragments were recloned into the vector pUC19 [116]. The recombinant plasmid was designated pJN37-19 (SLT-I) and pNN111-19 (SLT-II) [116].

Seventy-four Gram-negative bacterial isolates from the Michigan Department of Public Health and hospital laboratories, including *E. coli* 0157:H7 isolates with confirmation of verotoxin production by Center of Disease Control, were collected (table 4). *E. coli* EDL931 and HS were used as positive and negative controls respectively.

Frozen competent *E. coli* HB101 cells and plasmid pUC19 were purchased from Bethesda Research Laboratories (BRL), Gaithersburg, MD. The *E. coli* HB101 was transformed with pUC19 as a control. Transformation was performed by the calcium chloride procedure (Appendix I) [53,97].

A molecular weight standard, Hind III digested lamda phage, was purchased from International Biotechnologies Inc. New Heaven, CT. The labeled DNA probe carrier herring sperm DNA and yeast tRNA were purchased from Boehringer Mannhein and BRL.

Table 4. Isolates used in study

Test No.	Organism	Lab. No.	Test No.	Organism	Lab. No.
89y01	*E. coli*	SH1946	89y38	Hem[a] *E. coli*	1565-87
89y02	Hem[a] *E. coli*	3-85	89y39	*Shig. sonnei*	564-89
89y03	*E. coli*011ab:NM	VM25377-c1	89y40	Hem *E. coli*	1784-87
89y04	Hem[a] *E. coli*	2185-86	89y41	*E. coli*	SH1725
89y05	*S. newport*	509-89	89y42	Hem[a] *E. coli*	1896-87
89y06	Hem[a] *E. coli*	1396-85	89y43	*E. coli*	VMO11348C1
89y07	Hem[a] *E. coli*	1560-85	89y44	Hem[a] *E. coli*	51-88
89y08	*E. coli*	SH10468	89y45	*Shig. sonnei*	565-89
89y09	*S. tym.*05-neg.	542-89	89y46	Hem[a] *E. coli*	158-88
89y10	Hem[a] *E. coli*	1167-86	89y47	Hem[a] *E. coli*	469-88
89y11	*S. berta* (H_2S+)	543-89	89y48	Hem[a] *E. coli*	811-88
89y12	*Shig. sonnei*	549-89	89y49	Hem[a] *E. coli*	1080-88
89y13	Hem[a] *E. coli*	1385-86	89y50	*E. coli*	SH13133M
89y14	*E. coli*	SH15268	89y51	Hem[a] *E. coli*	1136-88
89y15	Hem[a] *E. coli*	1409-86	89y52	*Shig. boydii*-10	1028-87
89y16	Hem[a] *E. coli*	1427-86	89y53	Hem[a] *E. coli*	1161-88
89y17	*Shig. sonnei*	551-89	89y54	*S. montevideo*	568-89
89y18	Hem[a] *E. coli*	1476-86	89y55	*E. coli*	SH9097
89y19	*E. coli*	SH15955	89y56	Hem[a] *E. coli*	1223-88
89y20	*Shiga. boydii*-1	615-88	89y57	Hem[a] *E. coli*	1275-88
89y21	Hem[a] *E. coli*	1494-86	89y58	Hem[a] *E. coli*	1302-88
89y22	*S. enteritidis*	555-89	89y59	Hem[a] *E. coli*	1445-88
89y23	*E. coli* 026	H-30	89y60	Hem[a] *E. coli*	1552-88
89y24	Hem[a] *E. coli*	1582-86	89y61	*E. coli*	SH124
89y25	*S. typhimurium*	556-89	89y62	Hem[a] *E. coli*	1573-88
89y26	Hem[a] *E. coli*	1676-86	89y63	*Shig. dysent.*-6	1863-88
89y27	Hem[a] *E. coli*	1951-86	89y64	Hem[a] *E. coli*	1876-88
89y28	Hem[a] *E. coli*	2017-86	89y65	Hem[a] *E. coli*	1892-88
89y29	*E. coli*	SH2037	89y66	Hem[a] *E. coli*	87-89
89y30	*Shig. dysent.*-6	868-88	89y67	*E. coli* Rough	H-S
89y31	Hem[a] *E. coli*	2083-86	89y68	*E. coli*	SH4319
89y32	*S. thompson*	557-89	89y69	Hem[a] *E. coli*	325-89
89y33	Hem[a] *E. coli*	48-87	89y70	*S. hadar*	571-89
89y34	*S. heidelberg*	558-89	89y71	*E. coli*	SH4018M
89y35	Hem[a] *E. coli*	288-87	89y72	*Shig. sonnei*	58a1-89
89y36	Hem[a] *E. coli*	593-87	89y73	Hem[a] *E. coli*	354-89
89y37	Hem[a] *E. coli*	1510-87	89y74	*E. coli*	SH6783

a: hemorrhagic.

Chemicals and Reagents

Ampicillin, bovine serum albumin (fraction V), bromphenol blue, 50X Denhart's solution, dextran sulfate, ethylenediaminetetraacetatic acid (EDTA), Ficoll, formamide, heparin (from porcine intestinal mucosa), isoamyl alcohol, magnesium sulfate, N-laurylsarcosine, sodium phosphate, and tris-hydroxymethyl-aminomethane (Tris) were purchased from Sigma Chemical company, St. Louis, MO. Glacial acetic acid, hydrochloric acid, and sodium hydroxide were from Mallinckrodt, Paris, Kentucky. Chloroform (HPLC grade), lithium chloride, and ammonium acetate were from Aldrich, Milwaukee, WI. Phenol (redistilled nucleic acid grade), cesium chloride, and restriction enzyme HindIII and BamHI were products of BRL. Tris-HCL and proteinase K were products of Boehringer Mannheim (Indianapolis, IN). 100% ethanol (U.S. Industrial Chemicals, Tuscola, IL), agarose and ethidium bromide were purchased from IBI. Kodak GBX developer and replenisher, Kodak GBX fixer and replenisher, and Kodak indicator stop bath were purchased from Eastman Kodak Company, Rochester, NY. Other chemicals and reagents include sodium chloride (Fisher Scientific, Fair Lawn, NJ), ether (J.T. Baker Co., Phillipsburg, NJ), sodium citrate (MCB), P-ATP (Du Pont Company, Wilminton, DE), Pico-Fluor scintillation cocktail (Packard Instrument Company, Inc., Downers Grove, IL), and sodium dodecyl sulfate (SDS; Pierce, Rockford, IL).

Growth Media

S.O.C. (2% bacto-tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM$MgSO_4$, 20mM glucose) was used in *E. coli* HB101 transformations. LB (Luria-Bertani) antibiotic broth [10g/L bacto-

trypton (Difco, Detroit, MI), 5g/L bacto-yeast, 19g/L sodium chloride, and 50mg/L ampicillin] and agar plates were used in scaling up and culturing of *E. coli* HB101 with recombinant DNA. When preparing the colony blots, organisms were grown on TSA (Michigan Department of Public Health, Lansing, MI) agar plates.

Equipment

Eppendorf centrifuge 5415, Eppendorf pipetters (Brinkman, Westbury, NY), vortex (Scientific Industries, Boehmia, NY), 65°C and 42°C water bath (Precision, Chicago, IL), 37°C shaker bath, orbit shaker (Lab-Line, Melrose Park, IL), XT-400D top loading balance (Fisher Scientific), Centrifuge IEC B-20A (damon/IEC Division, Needham Heights, MA), IEC 870 rotor, Eppendorf tips, Eppendorf tubes (Cole-Parmer, Chicago, IL), Oakridge tubes, 0.45 μm 150 ml, 500 ml, and 1000 ml filter units (Nalgene, Rochester, NY), power supply (Hefer Scientific Instrument), Mayer nitrogen evaporator (Organomation, South Berlin, MA), microwave (Kenmore, Sears, Roebuck and Co., Chigaco, IL), NACS Prepac, combs, gel decks, H5, H6 electrophoresis systems (BRL), pH meter (Markson 90, Taiwan, ROC), Elutrap (electro-separation system, Schleicher & Schuell, Inc., Keene, NH), DMS 200 UV visible spectrophotometer (Varian Techtron Pty limited, Mulgrave, Victoria, Australia), RC 70-Ultracentrifuge (Sorvall Instrument, DuPont company, Wilmington, DE), Benchtop shield (Ann Arbor Plastics, Ann Arbor, MI), 2 ml polyallomer tubes (Kontron), Centricon 30 microconcentrator (Amicon Division, W.R.Grace & Co., Danver, MA), polaroid film type 47, No.2 red filter (Fabrigue aux EU par Polaroid Corp., Cambridge, MA), roundpoint toothpicks (Forster Mfg. Co., Wilton, Maine), sterile cotton swab (Cheeton, Dayville, CT), vacuum oven (National Appliance Company,

Heinicke Company, Portland, OR), stirrer/hot plate, desiccator, pyrex (Corning Glass Works, Corning, NY), microcooler (model 260011, Boekel Industries Inc.), PL-112 cordtrol heating mantle (Glas-Col Apparatus Company, Terre Haute, IN), liquid Scintillation systems LS 1801 (Beckman, Arlighton Heights, IL), Kodak adjustable safelight lamp (model B), Kodak diagnostic X-ray film (X-OMAT AR), and Kodak X-ray exposure cassette containing intensifying screen (Eastman Kodak Company), transilluminator (Michgan Dept. Public Health), polaroid camera (Polaroid Corp, Cambridge, MA).

Probe DNA Isolation

Modified Kado and Liu Rapid Plasmid Isolation Procedure To ensure the existence of recombinant plasmids in the *E. coli* strains provided (HB101), a rapid plasmid minipreparation was pursued. The Kado and Liu method [65] utilizes the molecular characteristics of covalently closed circular deoxyribonucleic acid that are released from cells under conditions that denature chromosomal DNA by using alkaline sodium dodecyl sulfate at elevated temperatures. Proteins and cell debris were removed by extraction with phenol-chloroform. This method permits the selective isolation of plasmid DNA that can be used directly in electrophoretic analysis, restriction endonuclease analysis, nick translation, transformation, and DNA cloning experiments.

The protocol is as follows: Bacterial strains were grown on LB (with ampicillin 50 μg/ml) medium, 37° C, overnight. A loopful of growth from the culture was suspended in a 1.5 ml microcentrifuge tube containing 200μl lysing solution and incubated for one hour at 65° C. The lysate was then extracted with an equal volume of phenol-chloroform, centrifuged for 5 min at 14,000 rpm, extracted twice with diethyl ether, evaporated under

nitrogen to remove trace amounts of ether, and transferred to a new 1.5 ml centrifuge tube. The plasmid DNA was precipitated by 800µl -20° C, 95% ethanol, after adding 100µl 3M ammonium acetate at -20° C for 30 min. Precipitated DNA was pelleted by centrifugation for 30 min at 14,000 rpm, 4° C, evaporated until dry under nitrogen, followed by resuspending of the DNA in 60µl TE buffer. (See Appendix III for reagent preparation)

Large Scale Kado and Liu Plasmid Isolation To scale up for DNA probe labeling, cell cultures were grown in 1 liter LB ampicillin broth (500 ml each in two 1 liter flasks) overnight on 37° C shaker. Overnight cultures were centrifuged at 6,000 rpm for 15 min, and the cells were resuspended in 90 ml TE buffer. The suspension was then transferred into six 50 ml Teflon tubes, and centrifuged at 6000 rpm for 10 min. After pouring off the supernatant, cells were lysed in 15 ml K&L lysing solution by placing in 65° C water bath for 1.5 hours. The lysate was extracted with 15 ml phenol-chloroform and centrifuged at 12,000 rpm for 30 min. The top layer was then transferred to a new 50 ml Teflon tube and the extraction was repeated again. After phenol-chloroform extraction, the TE saturated chloroform was used to rid the material of phenol contamination. The DNA was then centrifuged at 12,000 rpm for 30 min. The top layer was transferred to a polycarbonate tube. The plasmid DNA preparation was then neutralized by adding 4 ml of ammonium acetate and precipitated by adding 25 ml of 95% ethanol at - 70° C for 30 min. The DNA pellet was obtained by centrifuging at 15,000 rpm for 30 min, pouring off the supernatant and drying on a nitrogen evaporator. Finally, 200 µl dist. water was added to each tube to resuspend the DNA overnight [65].

Purification of Closed-Circular Plasmid The purification was

accomplished by centrifugation to equilibrium in a cesium chloride/ ethidium bromide gradient [3,93]. Because ethidium bromide unwinds DNA when it intercalates, less ethidium bromide can be bound by a covalently closed circular plasmid than can be bound by nicked plasmid or chromosomal DNA. Since binding of ethidium bromide to DNA lowers its density, the plasmid band appears at a higher density in an equilibrium density gradient [93].

To prepare the gradient, 1 ml of DNA solution (both pJN37-19 and pNN111-19) was placed in a 10 ml ultracentrifuge tube. Then 9 ml of sterile dist. water and 10 g of solid cesium chloride were added and completely dissolved in the solution by mixing gently. Finally, 0.8 ml of 10 mg/ml ethidium bromide was added and mixed. Sterile dist. water was used to fill the remainder of the tube. After mixing well, the capped tube was placed in a precooled titanium rotor and centrifuged at 60,000 rpm for 16 hours at 4° C. The second day, the tube was examined under UV light, and the closed circular plasmid (the lowest band) was collected through a #18 needle inserted into the side of the tube.

After the DNA was collected, isoamyl alcohol extraction was performed to remove the intercalated ethidium bromide. This was done by adding an equal volume of isoamyl alcohol, mixing vigorously, and centrifuging at 3,000 rpm for 3 minutes (repeated 5 times).

A Centricon microconcentrator was used to desalt the solution. This was performed by manufacture's instructions. After centrifugation, the DNA suspension was collected (100-150 µl per tube) and precipitated by adding 100 µl ammonium acetate, 800 µl cold ethanol, placing in a -20° C freezer overnight, and centrifuging at 14,000 rpm for 30 min at 4° C. The supernatant was discarded, and the pellet was dried under nitrogen and resuspended in 150 µl sterile water. The clean DNA preparation was then ready for enzyme restriction after determination of DNA concentration.

To determine the concentration of the DNA in suspension [98], 1 µl (or 2 µl) of preparation was mixed with 999 µl (or 998 µl) of sterile water in a 1 ml cuvette. The absorbance was read at 260 nm. Since 1 OD unit is equal to 50 µg/ml double-stranded DNA, an accurate concentration was then determined by calculation: absorbance × dilution factor × 50 µg/ml.

Digestion of DNA with Restriction Endonucleases It is generally accepted that one unit of restriction endonuclease corresponds to the amount of enzyme required to completely digest 1 µg of DNA in one hour of incubation under optimal assay conditions [95,130]. In this study, to ensure complete cutting, 3 units of restriction enzyme were used to digest 1 µg of DNA.

For screening, 10 µl of vector pUC-19 and 10 µl of pJN37-19 & pNN111-19 DNA from a minipreparation was mixed with 2.4 µl sterile dist. water, 1.6 µl 10X stock buffer, 1.6 µl BamHI for pUC-19 and pJN37-19, and 1.6 µl HindIII for pNN111-19 to make up the final volume of 16 µl in a 0.5 ml Eppendorf tube. The mixture was mixed thoroughly followed by incubation in a 37° C water bath for 2 hours.

For purification of probe DNA, 28 µg (decided by OD reading) of pJN37-19 and pNN111-19 was separately cut by adding 90 units (9 µl) of BamHI (for SLT-I) and HindIII (for SLT-II), 10 X buffer, and sterile dist. water to a 200 µl final volume. The mixture was incubated at 37° C overnight.

Agarose Gel Electrophoresis The standard method used to separate, and identify DNA fragments is electrophoresis through agarose gels [96]. In this study, agarose gels were prepared by suspending 300 mg agarose in 30 ml TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH:8.0) for a 1% minigel (5X7 cm) or 800 mg agarose in 80 ml TBE buffer for a

1% large (11X14 cm) gel. Agarose was melted in a microwave oven, mixed well and cooled to 45-50° C before pouring. The cooled agarose was poured in a taped gel deck and allowed to stand 30-60 minutes at room temperature for polymerization. DNA prepared from either the mini-preparation or from the large scale preparation was mixed with loading buffer (40% glycerol, 15% Ficoll, 0.25% bromophenol blue) in a 4:1 ratio. The mixture was then incubated in a 65° C water bath for 5 minutes before being loaded on the gel.

After gels were completely set, the comb and tape were carefully removed from the gels. The gels were mounted in the electrophoresis tank (horizontal apparatus, BRL Model H5, H6) and covered by TBE buffer to a depth of about 1 mm. Samples, mixed with loading buffer, were loaded into the slots of the submerged gels using a micropipette. All electrophoresis was carried out at 10 V/cm for 1.5 hours when using mini-gels or for 2.5 hours when using large gels. A molecular weight standard (hindIII digested lamda phage DNA) was resolved identically and used for estimation of the size of DNA fragments.

After electrophoresis, gels were stained in 4 μg/ml ethidium bromide solution for 15 minutes and DNA bands were visualized using a 302 nm UV transilluminator. Photographing was done by using polaroid type 47 film exposed through a Toshiba No. 2 red filter.

Recovery of DNA From Agarose Gels by Using ELUTRAP™ The DNA bands of interest were cut out from large agarose gels as close to the band as possible (by visualizing under UV light). ELUTRAP™ (S&S), an electro-separation system [46] was used in this study to recover probe DNA from agarose gels. The ELUTRAP™ device consists of a 1.2X10 cm sample chamber limited at each ends by an S&S BT1 membrane. The BT1 is an inert membrane with a dense matrix through which buffer ions

and molecules less than 3-5 Kd can pass under the influence of an electric field. A microporous membrane, the S&S BT2, acts as a prefilter that prevents agarose and other particulates from entering the purified sample. Together, the BT2 and BT1 membranes form a "trap" into which the samples migrate. The electric field acts as the driving force filtering the molecules through the membranes; when the voltage is switched off, the membranes seal the trap, preventing the diffusion of sample out of the trap.

The procedure was performed according to manufacture's instructions. The sliced gel was cut into half so the length of the gel was shorter than 6 cm, and then placed in the sample chambers. The chambers and devices were then filled with TBE buffer and the unused channels of the central tray were sealed off. A field strength of 200 volts was applied for two hours. After elution was completed, polarity was reversed by switching connecting wires for approximately 20 seconds at 200 volts to remove any material that may be attached to the BT1 membrane. The eluate was collected into a Eppendorf tube using a pasteur pipette. The ethidium bromide in DNA was then removed as previously described in the "Purification of closed-circular plasmid" section, and the DNA was precipitated. The final concentration of resuspended DNA was determined by spectrophotometry as previously described.

Probe DNA Labeling

Many different and reliable methods for labeling DNA fragments have been developed over the past few years to generate probes for the detection of DNA and RNA sequences by hybridization. Three kinds of labeling technique have been utilized in this study including 1) nick translation [139], 2) random primed DNA labeling [39], and 3) chemical

modification by sulfonation of the cytosine residue [163].

Nick Translation The mechanism of nick translation involves the use of limiting amounts of DNase I generating nicks in DNA and the combined activities of 5'→3'polymerase and 5'→3' exonuclease activities of *E. coli* DNA polymerase [5,132]. In this study, the probe DNA (SLT-I & II) was labeled with biotin by nick translation in the presence of biotin-7-dATP using the Bluegene^{TM} nonradioactive DNA detection system (BRL). The biotin-labeled nucleotide was incoporated into DNA by DNA polymerase I in the presence of the other three unlabeled deoxynucleoside triphosphates.

The components of the nick translation reagent system were purchased from BRL and the reaction was performed as follows. The reaction mixture including 5 µl of 0.2 mM dTTP, dCTP, and dGTP, 1 µg SLT-I or 1 µg SLT- II, 2.5 µl 0.4 mM biotin-7-dATP, and sterile distilled water was made to a total of 45 µl and pipetted into a 1.5 ml microcentrifuge tube sitting on ice. After mixing briefly, 5 µl DNA polymerase I and DNA Pol I/DNase I was added into the tube followed by thorough, gentle, mixing. The reaction was then carried out at 15° C for 90 minutes in a microcooler. At the end of the incubation, 5 µl of stop buffer and 1.25 µl of 5% (W/V) SDS were added to stop the reaction. To remove unincoporated nucleotides and to precipitate labeled DNA, the reaction mixture was mixed with 6 µl of 10 mg/ml yeast t-RNA, 6 µl of 4M $LiCl_2$, and 205 µl cold 95% ethanol, and incubated at -20° C for 1 hour, followed by 30 minutes centrifugation at 14,000 rpm, 4° C. The pellet was washed with cold 70% ethanol, dried under nitrogen, and resuspended in 40 µl of 0.1%SDS TE buffer at 37° C for 15 minutes. The biotin-labeled probes could be stored in solution at -20° C for at least 1 year.

Random Primered DNA Labeling The method of "random primed" DNA labeling developed by Feinberg and Vogelstein [39] is based on the hybridization of a mixture of all possible hexanucleotides to the DNA to be labeled. The complementary strand is synthesized from the 3'-OH termini of the random hexanucleotide primer using Klenow enzyme. In this study, modified deoxynucleosides triphosphate (biotin-dATP, digoxigenin-dUTP, or ^{32}P-dATP) were incoporated into the newly synthesized complementary DNA strand by using this DNA labeling technique. The random primed DNA labeling kit was purchased from Boehringer Mannhein and the protocol used was primerly according to the manufacture's instruction.

The probe DNA to be labeled was denatured prior to random primed labeling. This was done by heating for 10 minutes at 95° C and chilling quickly on dry ice. The following were then added into a microcentrifuge tube on ice: 1 μg of freshly denatured DNA, 2 μl of hexanucleotide mixture, dNTP labeling mixture (volume varied with different labeled-deoxynucleoside-triphosphate systems), made up to 19 μl with sterile redistilled water, and 1 μl Klenow enzyme. The reaction was executed for 30 minutes with ^{32}P labeling or overnight with nonradioactive labeling at 37° C, and stopped by adding 2 μl of 0.2M EDTA, pH 8.0 and incubating at 65° C for 10 minutes. The nonradioactive-labeled DNA was then precipitated by adding 2 μl of 10 mg/ml yeast t-RNA, 3 μl of 4M $LiCl_2$, 8 μl distilled water, and 100 μl of cold 95% ethanol, and incubating at -70° C for 30 minutes. Recovery of DNA was accomplished using the procedure described in the "nick translation" section. The ^{32}P-labeled DNA mixture was purified through NACS PREPAC column (BRL) to remove all of the unincoporated NTP (see Appendix IV).

Chemically Modified DNA This labeling technique is utilized in a Chemiprobe^{TM} kit purchased from FMC (FMC bioProducts, Rockland, ME). Labeling was accomplished by inserting an antigenic sulfone group into cytosine moieties of ssDNA [163]. The cytosines were sulfonated at carbon 6 by sodium bisulfite at high molarity. The resulting sulfone was stabilized by the substitution of the amine group on carbon 4 with a nulceophilic reagent such as methoxyamine. In this way, cytosines were transformed into N-4-methoxy-5,6-dihydrocytosine-6-sulfonate.

The DNA modification procedure was accomplished completely by following manufacture's instruction: 50 µl (25 µg) salmon sperm DNA, provided with the kit as a control, or 1 µg (diluted with water to make up 50 µl) of probe DNA (SLT-I & SLT-II) was denatured by boiling for 5 minutes and quickly chilled in a microcentrifuge tube on ice. Then 25 µl of modification solution A was added to the tube and mixed gently followed by adding 6.25 µl of modification solution B. The ratio of chemicals in the reaction mixture was maintained at 1 vol DNA: 1/2 vol solution A: 1/8 vol solution B. The modification mixture was incubated overnight at room temperature. The modification chemicals were removed from the sulfonated DNA by ethanol precipitation as follows: 1 µl of 10 mg/ml t-RNA and 2.5 volumes of ice cold 95% ethanol was added to the mixture; after thorough mixing, the tube was incubated at -70° C for 30 minutes, and centrifuged at 14,000 rpm at 4° C for 30 minutes; the supernatant was decanted and the pellet was dissolved in 40 µl TE buffer.

Determination of Recovery of Labeled DNA To determine the volume of labeled DNA suspension to be put into the hybridization solution, it was important to estimate the labeled DNA concentration. To do this for ^{32}P-labeled DNA, 1µl of labeled DNA was taken into 4 ml of scintillation cocktail, mixed, and the activity in the liquid scintillation

system was read for 1 minute.

For nonradioactive-labeled DNA, 1μl of labeled DNA was serially diluted in DNA dilution buffer. The dilutions were spotted on either a nylon or a nitrocellulose membrane strip. The strips were then dried in a vacuum oven for 2 hours at 80°C. The procedure was the same as that for test strips in each nonradioactive DNA detection system. The strips spotted with labeled probe DNA dilution were then developed (see the section on "target DNA detection") along with DNA detection test strips to estimate recovery of labeled DNA by color development.

DNA Blot Preparation

Blotting Membrane Several kinds of blotting membranes were used in this study in order to search for the best performance in different DNA detection systems. The membranes used include nitrocellulose (Bethesda Research Laboratories, gaithersburg, MD), Nytran™ nylon (Schleicher & Schuell, keene, NH), nylon-GTG™ and pure nitrocellulose (FMC BioProducts, Rockland, ME), Zeta-probe™ nylon (Bio-Rad Laboratories, Richmond, CA), Whatman #541 (Whatman Limited, England), and Immobilon-P™ (Millipore Corporation, BedFord, MA). Baby blot kits purchased from BRL were used for Southern blotting, and S&S #470 absorbent paper and Whatman 41 were used in the colony blotting procedure.

Southern Blotting To ensure the specificity of DNA probes, a southern blot hybridization was performed prior to colony blot hybridization [4,8,159]. Southern blotting was accomplished by transferring DNA from a mini- gel to a nitrocellulose membrane. The mini-gel, after staining and photographing, was transferred to a washing

dish. The gel was depurinated, denatured and neutralized as follows: (1) depurination: 100 ml 0.25 M HCl was added to the washing dish for 20 minutes with gentle agitation, the gel was sequentially rinsed with distilled water; (2) denaturation: 100 ml of 0.5 M NaOH, 1 M NaCl was added to the washing dish for 20 minutes with gently agitated. The solution was then decanted and the gel was rinsed with distilled water; (3) neutralization: 100 ml of 0.5 M Tris-HCl, 3 M NaCl, pH 7.4 was added to the washing dish with gentle agitation for 30 minutes. Transferring of DNA was performed by stacking three blotting pads in the bottom of a container, saturating the pads with 40 ml of 20X SSC, placing the gel and a sheet of wicking paper onto the saturated blotting pads, and sequentially placing a sheet of prewet nitrocellulose membrane, a wicking sheet, a stack of 7 blotting pads, a container lid loaded with 200 gm weight over the top of gel for overnight. After transferring, the nitrocellulose membrane was washed in 2X SSC for 5 minutes [4], and dried on a piece of filter paper, followed by placing the dried membrane between 2 new pieces of filter paper and baking for 2 hours under vacuum at 80° C.

Bacterial Colony Blotting Colony blots were prepared either on Whatman #541 or on nitrocellulose and nylon membranes. For colony blots using Whatman #541, the protocol followed was that of Maas [91]: Bacterial colonies were grown on TSA agar plates at 37° C overnight and oriented according to the grids beneath the plates. The following day, a piece of Whatman paper was placed over the inoculated plate with an arrow between colonies 1 and 2, and any trapped air bubbles were removed with tweezers. After two hours, the papers were then transferred to a pyrex petri dish containing S&S absorbent paper #470 (colony side up) saturated with lysing solution (0.5 M NaOH, 1.5 M NaCl) and steamed for 3 minutes. After removal from the steamer, the papers were

immersed in fresh lysing solution for 1 minute, neutralization solution (1 M Tris-HCl, 2 M NaCl, pH 7.0) for 5 minutes, 2X SSC for 5 minutes, blotted on filter paper, and air dried at 37° C.

For colony blots on nitrocellulose or nylon membrane [49,50,164], the blots were prepared either by transferring the colonies from a master plate to a membrane or by inoculating colonies directly to a sterile membrane placed on an agar plate. When using the former method, bacterial organisms were picked with sterile toothpicks, and inoculated to TSA agar plates oriented according to the grid beneath the plates. For the latter method, streaked membranes (either nitrocellulose or nylon) were boiled in distilled water for 1 minute, and autoclaved before placing on TSA plates. Bacterial colonies were then directly grown on the membrane by inoculating with sterile toothpicks. Plates were incubated at 37° C for 6 hours or at 25° C for 16-18 hours until a 1-2 mm diameter colony size was reached. Before lysing the cells, the plates were incubated at 4° C for at least 30 minutes. Lysis was then performed as follows: membranes were transferred (colony side up) onto S&S absorbant paper #470 saturated with 0.5 M NaOH, 1.5 M NaCl in a pyrex petri dish, incubated for 15 minutes, and blotted on a sheet of filter paper to remove excess fluid. Membranes were then sequentially placed in 0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5 for 15 minutes, immersed in 2X SSC for 5 minutes, air dried, and baked under vacuum at 80° C for 2 hours. Blots were stored in a dessicator prior to use.

Prehybridization, Hybridization, and Posthybridization [9,13,41,116]

All colony blots used for nonradioactive DNA detection systems were pretreated with proteinase K [41,76,145,176] to get rid of cell debris before prehybridization. Membranes were rinsed in 2X SSC to wet them

thoroughly, followed by incubating in 500 ug/ml proteinase K solution (2X SSC, 0.1% SDS, 20 ml/100cm^2) at 37° C in a water bath agitated at 100 rpm for 1 hour. After incubation, the membranes were removed to a washing tray and washed with 2X SSC twice, 5 minutes each time. During the washing, a sterile cotton swab rinsed with 2X SSC was used to further clean off any cell debris or dirt remaining on the membranes. Excess fluid was drained out and membranes were then ready for prehybridization.

The membranes to be prehybridized were placed in a hybridization bag back-to-back if there was more than one membrane. The bag was cut so that it was only slightly larger than the membrane. Prehybridization solution was then added to the bag (50 ul/cm^2), and any trapped air was removed before the bag was sealed. Prehybridization was performed at 42° C for 4 hours occasionally redistributing the solution in the bag.

At the end of the prehybridization, hybridization solution was prepared by adding labeled probe DNA (SLT-I or SLT-II) to hybridization solution (25 ul/cm^2) so that the final concentration (activity) of labeled probe DNA in the solution was 150 ng/ml for Bluegene™, 100 ng/ml for Genius™ and Chemiprobe™, and 1,000,000 cpm/ml for the radioactive detection system (^{32}P). The probe DNA was then denatured by boiling the solution in water for 10 minutes and quickly chilling in ice. The prehybridization solution was emptied by cutting a corner of the hybridization bag and the hybridization solution was added to the bag. Hybridization was performed at 42° C overnight (16-20 hrs). Nonradioactively-labeled-DNA hybridization solution could be used many times.

After hybridization, membranes were washed twice in solution I (250 ml/100cm^2, 2X SSC/0.1% SDS) and twice in solution II (250 ml/100cm^2, 0.2X SSC/0.1% SDS), 5 minutes each, at room temperature, and then twice in solution III (250 ml/100cm^2, 0.1X SSC/0.1% SDS), 20 minutes each, at 60-65° C. (See Appendix V for reagent preparation)

Target DNA Detection

Nonradioactive Systems The three different nonradioactive systems used in this study all utilize an enzyme, alkaline phosphatase, which converts a soluble chromogenic substrate system (NBT-BCIP) into an insoluble dye (See Appendix VI for buffer composition). The colored product indicates the presence of our target DNA [9,13,41].

"BlueGENE™" Test strips and membranes after the final posthybridization wash were washed for one minute in buffer 1 at room temperature and blocked for one hour in buffer 2 at 65°C. In a siliconized glass tube, 1 µg/ml streptavidin-alkaline phosphatase conjugate was diluted in buffer 1 at a 1:1000 ratio. Membranes were then incubated in the conjugate solution (7 ml/100cm^2) for 20 minutes with gentle agitation at room temperature, followed by washing the membranes X2 for 15 minutes in buffer 1 and once for 10 minutes in buffer 3 with gentle agitation. In a hybridization bag, the membranes were then incubated and developed with freshly prepared dye solution (7.5 ml/100cm^2) for 30 minutes to 3 hours (whenever the reaction was at maximum and background started showing). The reaction was stopped by buffer 4.

"Genius™" Test strips and membranes after the final posthybridization wash were washed for one minute in buffer 1 at room temperature and blocked for 30 minutes in buffer 2 (100 ml/cm^2) at room temperature. In a siliconized glass tube, anti-digoxigenin-Fab-fragment-alkaline phosphatase conjuagte was diluted in buffer 1 at a 1:5000 ratio so the final concentration was 150 mU/ml. Membranes were then incubated in the conjugate solution (20 ml/100cm^2) for 30 minutes with gentle agitation at room temperature, followed by washing the membranes X2 for

15 minutes in buffer 1 and for 10 minutes in buffer 3 with gentle agitation. In a hybridization bag, the membranes were then incubated and developed with freshly prepared dye solution (10 ml/100cm^2) for 30 minutes to 24 hours (whenever the reaction was at maximum and background started showing). The reaction was stopped by washing the membranes in buffer 4 for 5 minutes.

"**Chemiprobe^{TM}**" The test strip was rehydrated in 1X SSC and the wet membranes, after the final posthybridization wash, were incubated with blocking solution I (25 µl/cm^2) in a hybridization bag for one hour at room temperature. At the end of one hour, the mouse monoclonal anti-modified DNA antibody was added into the bag at a dilution of 1:250, and the bag was resealed for one more hour. The membranes were then washed 5X for 10 minutes each with wash solution (3 ml/cm^2) before being stored overnight at 4° C in phosphate buffered saline (PBS). The second day, the alkaline phosphatase anti-mouse immunoglobµlin conjugate was diluted 1:250 in blocking solution II and added in a bag with membranes. For high sensitivity, the incubation was performed for 3 hours, and the membranes were washed 5X for 10 minutes each in washing solution. The washed membranes were then transferred to a new plastic bag filled with freshly prepared dye solution (50 µl/cm^2) and incubated at 37° C up to 3 hours or until the reaction was at maximum and the background started showing. The color development was stopped by washing the nylon membrane for 10-20 seconds in 95% ethanol, followed by a rinse in dist. water or by washing the nitrocellulose membrane in dist. water only.

Radioactive System Following final washing membranes were placed on a piece of absorbent paper to remove excess fluid and then wrapped in Saran Wrap to keep them moist and to prevent contamination. The wrapped membranes were then put in an exposure cassette containing an intensifying screen with X-ray film. The film was exposed for 12-16 hours at -70° C [35,51,99]. The second day, the cassettes were brought to room temperature for 30 minutes and the film was developed for 3 minutes in developer, 30 seconds in stop bath with constant gentle agitation, 4 minutes in fixer with gentle agitation every 30 seconds, and 5 minutes in running water.

RESULT

Plasmid DNA Isolation

Recombinant plasmids, pJN37-19 and pNN111-19 in *E. coli* HB101 provided by Newland and Neill, and vector plasmid pUC19 in transformed *E. coli* HB101 were isolated by minipreparation. The isolated plasmids were digested with restriction endonucleases followed by electrophoresis. The results of electrophoresis demonstrated a successful transformation reaction by the existence of a band (lane #7 of figure 1.A) of DNA fragment located at the 2.7 kb position (Figure 1.B), which is very close to the actual size 2.686 kb of the plasmid pUC19. Two DNA fragments digested from pJN37-19 represented the vector plasmid pUC19 and the insert SLT-I genes located at the position of 2.7 kb and 1.1 kb (see: lane #3 of figure 1.A and figure 1.B), while two DNA fragments digested from pNN111-19 represented the vector plasmid pUC19 at 2.7 kb position and the insert SLT-II genes at 0.8 kb position on the gel (see: lane #5 of figure 1.A and figure 1.B). The actual sizes of SLT-I and SLT-II insert genes are 1.142 kb and 0.842 kb. The plasmid minipreparation confirmed the existence of probe DNA. The cell culture was then scaled up.

The concentration of recombinant plasmids purified from large scale preparations was 0.38 μg/μl for pJN37-19 and 1.4 μg/μl for pNN111-19 as determined by OD reading. Six μg of SLT-I and SLT-II probe genes on the agarose were recovered through ELUTRAP after 22.8 μg of pJN37-19 and 28 μg of pNN111-19 were digested and the DNA fragments were

separated by electrophoresis (figures 2.A&B). This indicated that the recovery of probe DNA was approximately 88% for SLT-I and 90% for SLT-II based on the following calculation:

SLT-I contained in digested pJN37-19 =

22.8 μg× 1.142 (kb)/3.828 (kb) = 6.8 μg

6 μg/6.8 μg × 100% = 88%

SLT-II contained in digested pNN111-19 =

28 μg× 0.842 (kb)/3.528 (kb) = 6.68 μg

6 μg/6.68 μg × 100% = 90%.

Efficiency of Labeling and Recovery of DNA Probe

According to the color reactions of serial-diluted, labeled-DNA on the dot blots, it was shown that ~160 ng of biotin-DNA probe was recovered from 1 μg of probe DNA by labeling in the nick translation reaction, precipitating with ammonium acetate and 95% ethanol, and resuspending in pure TE buffer. This suggested a low efficiency of labeling or recovery of DNA probe at 16%. The efficiency was improved to 40% by labeling the DNA using the same labeling reaction condition but precipitating the labeled biotin-DNA by adding t-RNA, $LiCl_2$, 95% ethanol, and finally resuspending the biotin-DNA in TE buffer containing 0.1%SDS followed by incubation at 37° C for 15 minutes. This modified procedure was adopted from the Technical Update for the Genius™ system (Table 5) [14]. The random-priming technique was also applied to biotin-DNA labeling, but the labeling efficiency of DNA was always ≤10% regardless of precipitating and resuspending protocols (Figure 3.A). However, the modified protocol for recovering nonradioactive labeled-DNA did help to increase recovery of the digoxigenin-DNA labeled by the random-priming

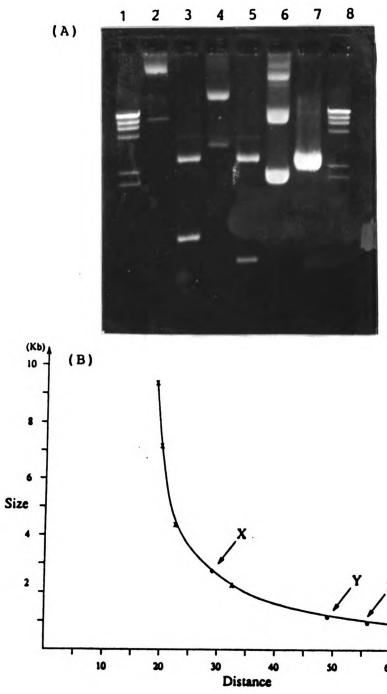


Figure 1 (A) Recombinant and vector plasmids isolated by minipreparation. 1% agarose gel, 10 v/cm, 1.5 hours. Lane #1: molecular weight standard-HindIII digested lamda phage, #2: pJN37-19, #3: pJN37-19 digested with BamHI, #4: pNN111-19, #5: pNN111-19 digested with HindIII, #6: pUC19, #7: pUC19 digested with BamHI, #8: standard. (B) Estimation of size of linear DNA fragments: by comparison with molecular standard. Size of DNA fragment vs. distance of migration of DNA fragments. X: pUC19, 2.7 kb; Y: SLT-I, 1.1 kb; Z: SLT-II, 0.8 kb.

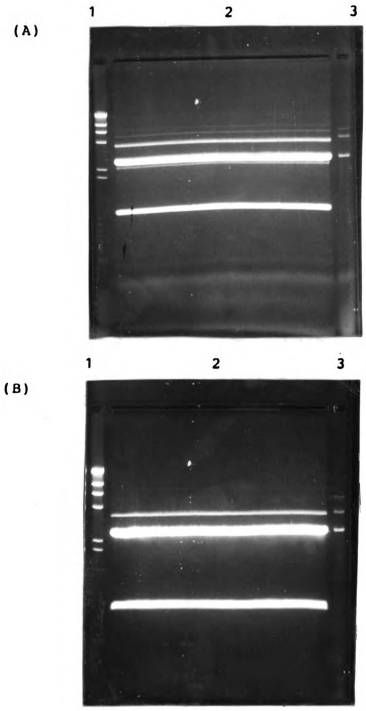


Figure 2. Large scale separation of SLT probe DNA from recombinant plasmid. 1% agarose, 10 v/cm, 2.5 hours. (A) SLT-I Lane #1: molecular weight standard-HindIII digested lamda phage, #2: 22.8 μg of pJN37-19 digested with BamHI, #3: pUC-19 digested with BamHI. (B) SLT-II Lane #1: molecular weight standard-HindIII digested lamda phage, #2: 28 μg of pNN111-19 digested with HindIII, #3: pUC-19 digested with BamHI.

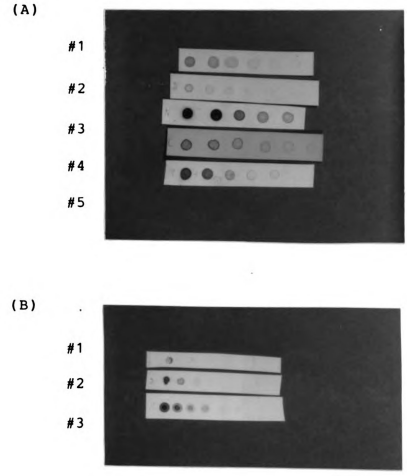


Figure 3. The color development of serial-diluted nonradioactive DNA probe on dot blots. (A) Biotin-DNA #1. Standard: left→ right 20, 10, 5, 2, 1, 0 pg; #2. DNA probe labeled by nick translation and recovered by the unmodified method: left→ right 2×10^2X, 4×10^3X, 1×10^4X, 2×10^4X, 4×10^4X, 1×10^5X, 2×10^5X; #3. DNA probe labeled by nick translation and recovered by the modified method: left→ right 20X, 100X, 1000X, 5000X, 1×10^4X; #4. DNA probe labeled by random priming and recovered by unmodified method: left→ right 250X, 500X, 1250X, 2500X, 5000X, 12500X; #5. DNA probe labeled by random priming and recovered by modified method: left→ right 20X, 100X, 1000X, 5000X, 1×10^4X, 1×10^5X. Color reactions were developed for one hour. **(B) Digoxigenin-DNA** #1. Standard: left→ right 10 pg, 1pg, 0.5 pg. 0.1 pg, 10 fg; #2. DNA probe labeled by random priming and recovered by the unmodified method: left→ right 10X, 100X, 1000X, 1×10^4, 1×10^5, 1×10^6; #3. DNA probe labeled by random priming and recovered by the modified method: left→ right 10X, 100X, 500X, 1000X, 5000X, 1×10^4. Color reactions were developed for 16 hours.

reaction. The efficiency was raised from ≤10% to ~40% (Figure 3.B). To get maximal labeling, an 18-20 hour incubation was required. The production of sulfonated-DNA by the chemical modification technique, performed following manufacturer's instructions, gave a satisfactory efficiency (>30%).

In the radioactive system, the SLT-I and SLT-II probe DNAs were labeled with ^{32}P-ATP by the random-priming reaction for 30 minutes. After removing unincorporated nucleotide, 1 µl of a 250 µl elution was taken to the scintillation counter to check activity. One µl of SLT-I probe elution gave 22,378 cpm activity, whereas 1 µl of SLT-II probe elution gave 62,440 cpm activity.

Table 5: Procedures of Recovery of Nonradioactive DNA probes

	Unmodified	Modified
Precipitation	Ammonium acetate + 95% ethanol	LiCl + tRNA + 95% ethanol
Resuspension	TE buffer, at RT	TE buffer containing 0.1%SDS, at 37° C

Preparation of Bacterial Colony Blots by Different Procedure

Bacterial colony blots were prepared by inoculating the bacterial colonies directly on sterile membranes placed on culture medium plates or by transferring the colonies from a master plate to membranes. The results

of this study (figure 4) demonstrated the former method provided higher contrast of color reactions between the positive controls and the negative controls in the BlueGENE™ nonradioactive DNA detection system (biotin-DNA probe). The background binding on negative controls were sometimes too strong to distinguish from positive controls.

Performance of Various Blotting Membranes with Different DNA Detection Systems

Five kinds of membranes were examined in this study. Immoblin-P™ membrane was found unsuitable for bacterial colony blotting because the membrane would not let lysing solution filter well. It also retained the pink dye in the MacConkey medium, which interferes with reading results. Zeta-Probe™, a nylon membrane, demonstrated serious nonspecific binding over the entire membrane in BlueGENE™ and Genius™ systems on both Southern and colony blotting. Therefore, only Whatman #541 filter membrane, Nylon-GTG™, nitrocellulose membranes of BRL and FMC, and Nytran™ nylon membranes were further tested in the three nonradioactive DNA detection systems.

With the BlueGENE™ system, Nylon-GTG™ membranes gave the most satisfactory results by providing the best contrast between the color reaction of positive controls and of negative controls, and no nonspecific binding (high signal-to-noise ratio). Other kinds of nitrocellulose and nylon membranes gave similar results: some weak background on negative controls but no nonspecific binding. The results of Whatman #541 filter membrane were unacceptable because the background on each spot made reading extremely difficult. Figure 5 demonstrates the results of different kinds of membranes.

In the Genius™ system, Nytran™ nylon membranes, and nitrocellulose

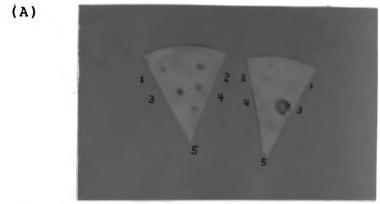


Figure 4. Comparison of different procedures for bacterial colony blotting. Method: (A) Transferred colonies from a master plate. (B) Colonies grown directly on membranes. Colony orientation: 1: *E. coli* HB101 with recombinant plasmid pJN37-19, 2: *E. coli* HB101 with recombinant plasmid pNN111-19, 3: *E.coli* HS, 4: *E. coli* EDL931, 5: *E. coli* HB101 with vector plasmid pUC19. Membranes were hybridized by biotin-SLT-II DNA probe and developed with BlueGENE™ nonradioactive DNA detection system.

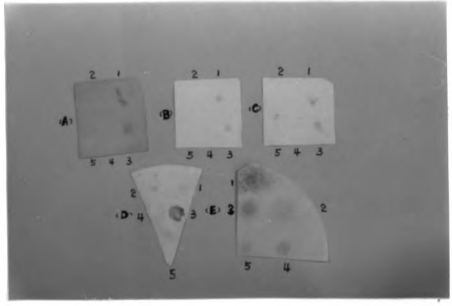


Figure 5. Performance of different kinds of membranes with the BlueGENE™ nonradioactive DNA detection system. Colony orientation 1: *E. coli* HB101 with recombinant plasmid pJN 37-19, 2: *E. coli* HB101 containing recombinant plasmid pNN111-19, 3: *E. coli* EDL 931, 4: *E. coli* HS, 5: E. coli HB101 with plasmid pUC19. (A) Nylon-GTG™ membrane (B) Nitrocellulose membrane of BRL (C) Nitrocellulose membrane of FMC (D) Nytran™ nylon membrane (E) Whatman #541 filter membrane. The membranes were hybridized with the SLT-I DNA probe.

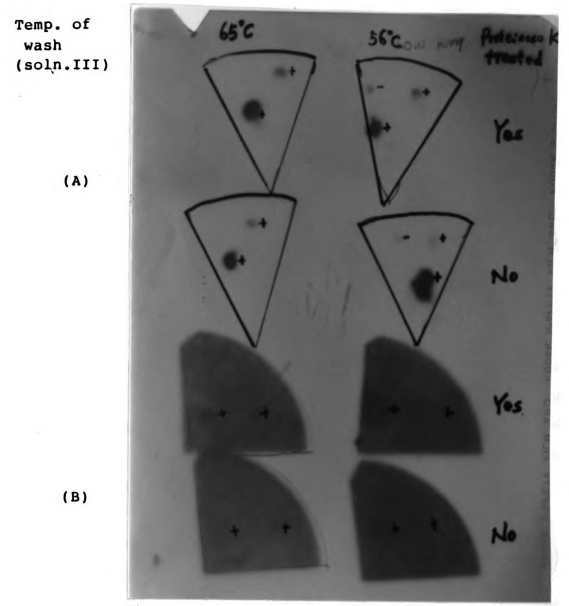


Figure 6. Performance of different membranes with ^{32}P-DNA radioactive detection system. (A) Nytran™ nylon membrane, (B) Whatman #541 filter membrane. +: positive controls.

membranes of BRL and FMC gave good results showing high contrast color reactions between positive and negative controls. Nylon-GTG™ membrane produced a little nonspecific binding but the positive and negative reactions were distinguishable. Whatman #541 filter membrane, again, produced very serious nonspecific binding and background problems.

In the Chemiprobe™ system, Nytran™ nylon membranes and Nylon-GTG™ membranes of FMC demonstrated the best results with a high positive-to-negative contrast and a higher binding capacity than those produced by nitrocellulose membranes of BRL and FMC. Whatman #541 filter membrane also showed promise with no background although a little nonspecific binding was evident.

In the ^{32}P-DNA radioactive detection system, only Whatman #541 filter membranes and Nytran™ nylon membranes were tested. The Nytran™ nylon membranes were found to have much higher binding capacities than Whatman #541 filter membranes. This allowed use of higher stringency washings and maintenance at a high signal-to-noise ratio. Whatman #541 filter membranes, however, produced nonspecific binding which made them difficult to read (Figure 6).

Hybridization Condition

Membranes not treated with proteinase K prior to prehybrdiztion persistently produced background problems in two nonradioactive DNA detection systems, BlueGENE™ and Genius™ systems (Chemiprobe™ system was not checked in this study). However, these false positive reactions were eliminated by treating the colony blots with proteinase K to clean the cell debris remaining on the blots. The proteinase K treatment made no difference in the radioactive DNA detection system (Fig.6).

When Nylon-GTG™ membranes were used with the BlueGENE™, addition of 5% dextran sulfate and 0.5% SDS in both prehybridization and hybridization solutions helped decrease nonspecific binding.

The concentration of probe DNA in the hybridization solution was also critical to the sensitivity of detecting target DNA. In the BlueGENE™ system, 160 ng/ml (manufacturer's suggesting range: 100-500 ng/ml) of probe DNA was found to give sufficient sensitivity. In the Genius™ system, however, the use of the manufacturer's suggested concentration (26 ng/ml) did not give satisfactory results. The concentration was increased to at least 100 ng/ml at which point satisfactory results were obtained. In the Chemiprobe™ system , a concentration of 100 ng/ml gave satisfactory sensitivity.

The temperature of posthybridiztion, especially the washing step using solution III, was critical for both the specificity and sensitivity. This was clearly demonstrated in the radioactive detection system (figure 6). When 55° C washing temperature was used, the background still existed, but disappeared when a 65° C wash temperature was used. More stringent conditions, such as washing above 68° C, would wash out all binding.

Results of Bacterial Colony Screening

The colony blots of 74 test and control organisms were screened with the BlueGENE™ and the Chemiprobe™ nonradioactive DNA detection systems on Nylon-GTG™ membranes, and with the ^{32}P-DNA radioactive detection system on Nytran™ membrane. The results of probing with each system demonstrated completle consistency with toxin-production as reported by the Center of Disease Control. The sensitivity and the specificity of each system as compared to the Verotoxin assay was 100%. The results are listed in Figure 7 and Table 6&7.

6	5	4	3	2	1	
13	12	11	10	9	8	7
20	19	18	17	16	15	14
27	26	25	24	23	22	21
34	33	32	31	30	29	28
40	39		38	37	36	35
#1	C5	C4	C3	C2	C1	

46	45	44	43	42	41	
53	52	51	50	49	48	47
60	59	58	57	56	55	54
67	66	65	64	63	62	61
74	73	72	71	70	69	68
#2	C5	C4	C3	C2	C1	

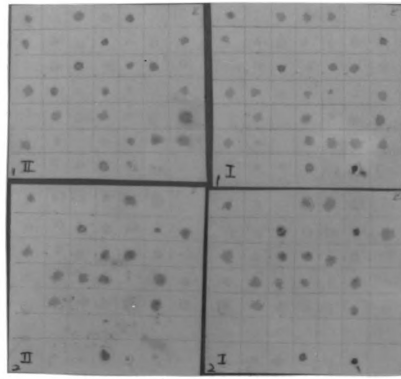
C1: E. coli HB101 with pJN37-19

C2: E. coli HB101 with pNN111-19

C3: E. coli EDL931

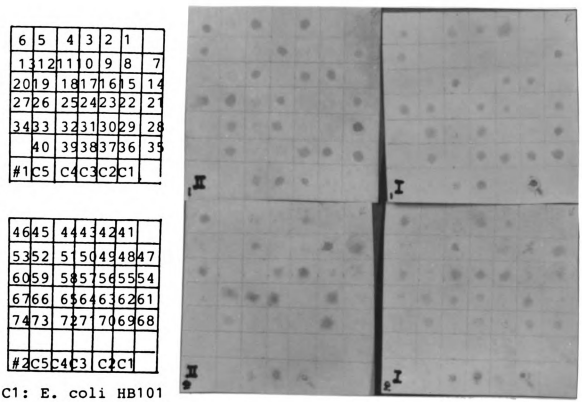
C4: E. coli HS

C5: E. coli HB101 with pUC19



(II) (I)

Figure 7(A). Results of bacterial colony screening using the BlueGENE™ nonradioactive DNA detection system on Nylon-GTG™ membranes. I: Hybridized with the SLT-I probe. II: Hybridized with the SLT-II probe. (Note: The organisms no. 44, 49, 53, 57, 59 have been retested because they were later found contaminated.)



C1: E. coli HB101 with pJN37-19

C2: E. coli HB101 with pNN111-19

C3: E. coli EDL931

C4: E. coli HS

C5: E. coli HB101 with pUC19

(II) (I)

Figure 7(B). Results of bacterial colony screening using the Chemiprobe™ nonradioactive DNA detection system on Nylon-GTG™ membranes. I: Hybridized with the SLT-I probe. II: Hybridized with the SLT-II probe. (Note: The organisms no. 44, 49, 53, 57, 59 have been retested because they were later found contaminated.)

Table 6. Results of Screening of SLT-producing in Different DNA Detection Systems and Verotoxin Assay

Strain no.	(A)	(B)	(C)	(D)	Strain No.	(A)	(B)	(C)	(D)
1	-	-	-	-	38	+	+	+	+
2	+*	+*	+*	+	39	-	-	-	-
3	+	+	+	+	40	+*	+*	+*	+*
4	+*	+*	+*	+	41	-	-	-	-
5	-	-	-	-	42	+*	+*	+*	+*
6	+*	+*	+*	+	43	+	+	+	+
7	+*	+*	+*	+	44	+	+	+	+
8	-	-	-	-	45	-	-	-	-
9	-	-	-	-	46	+*	+*	+*	+*
10	+a	+a	+a	+	47	+*	+*	+*	+*
11	-	-	-	-	48	+*	+*	+*	+*
12	-	-	-	-	49	+*	+*	+*	+*
13	+*	+*	+*	+	50	-	-	-	-
14	-	-	-	-	51	+*	+*	+*	+*
15	+*	+*	+*	+	52	-	-	-	-
16	+*	+*	+*	+	53	+*	+*	+*	+*
17	-	-	-	-	54	-	-	-	-
18	+*	+*	+*	+	55	-	-	-	-
19	-	-	-	-	56	+*	+*	+*	+*
20	-	-	-	-	57	+	+	+	+
21	+*	+*	+*	+	58	+	+	+	+
22	-	-	-	-	59	+*	+*	+*	+*
23	+	+	+	+	60	+*	+*	+*	+*
24	+*	+*	+*	+	61	-	-	-	-
25	-	-	-	-	62	+*	+*	+*	+*
26	+*	+*	+*	+	63	-	-	-	-
27	+*	+*	+*	+	64	+*	+*	+*	+*
28	+*	+*	+*	+	65	+*	+*	+*	+*
29	-	-	-	-	66	+*	+*	+*	+*
30	-	-	-	-	67	-	-	-	-
31	+*	+*	+*	+	68	-	-	-	-
32	-	-	-	-	69	+*	+*	+*	+*
33	+*	+*	+*	+	70	-	-	-	-
34	-	-	-	-	71	-	-	-	-
35	+*	+*	+*	+	72	-	-	-	-
36	+*	+*	+*	+	73	+*	+*	+*	+*
37	+*	+*	+*	+*	74	-	-	-	-

(A) BlueGENE™ (B) Chemiprobe™ (C) ^{32}P-DNA (D) Verotoxin assay. Results provided by CDC for test strains No.1-36 only confirm SLT production without distinguishing SLT-I or SLT-II.

*: Test strain had both SLT-I & SLT-II positive.

+: Test strain only has SLT-I positive (not include lane (D)).

a: Test strain only has SLT-II positive.

Table 7. Comparison of Results of Screening SLT-producing by DNA Detection Systems and Verotoxin Assay

(A)

		Verotoxin Assay	
		+	-
BlueGENE™	+	43	0
	-	0	31

(B)

		Verotoxin Assay	
		+	-
Chemiprobe™	+	43	0
	-	0	31

(C)

		Verotoxin Assay	
		+	-
^{32}P-DNA	+	43	0
	-	0	31

DISCUSSION

In 1982 a multistate outbreak of hemorrhagic colitis drew attention to an unusual clinical syndrome of diarrheal disease and its causative organism *E. coli* serotype O157:H7; more epidemic and sporadic cases have been reported since then. Laboratory diagnosis of hemorrhagic colitis is based on the demonstration of cytotoxins (Shiga-like toxin I and Shiga-like toxin II) produced by *E. coli* which is highly toxic for Vero and Hela cells. These antibody-dependent assays are inconvenient to use, especially when screening large numbers of isolates. DNA hybridization with probes derived from virulence genes has previously been demonstrated to be an alternate technique which can facilitate detection of bacteria containing similar pathogenic characteristics. However, these probes are routinely labeled with radioisotopes which are not considered to be suitable in all diagnostic laboratories. The establishment of nonradioactively labeled probes for screening large numbers of isolated bacterial colony blots would be, therefore, highly desirable. In this study, a set of DNA probes which have been developed to the genes for Shiga-like toxin were evaluated for identifying EHEC (especially serotype O157:H7) by nonradioactive detection. The parameters that influence the sensitivity and specificity of nonradioactive DNA probe hybridization for the detection of a target DNA sequence in colony blots were assessed in order to set up a reliable, safe, convenient, highly sensitive, and highly specific laboratory screening method for EHEC.

Improving the Efficiency of Recovery of Nonradioactive labeled-DNA Probe

Digoxigenin-labeled DNA was prepared by the random-primed DNA labeling techniques. Before recovering labeled-DNA with modified protocols, this procedure suffered from a very low efficiency of digoxigenin-labeled DNA recovery. The hydrophobicity of digoxigenin makes it necessary to exercise certain precautions to maximize the recovery of labeled-DNA [14]. Yeast tRNA was added during the ethanol precipitation steps as a carrier and, because digoxigenin-labeled DNA is somewhat more difficult to resuspend than unmodified DNA, TE buffer containing SDS was used to resuspend the precipitated DNA pellet by heating. The heat and the addition of SDS helped resuspend the DNA pellet by partially overcoming the hydrophobicity. These modifications did improve the yield of digoxigenin-labeled DNA. Ammonium acetate is not recommanded to precipitate nonradioactive-labeled DNA [14,41]. Its use results in a pellet, which is very difficult to resuspend. Nick translation was also utilized in this system [14], however, its ability to incorporate label was not as efficient as that obtained from random-primed labeling.

Using the BlueGENE^{TM} system, nick translation was the preferred labeling method when compared to random-primed labeling. This suggests that the labeling method, random priming vs.nick translation, may be system-, DNA- (ex. DNA sequence and structure), or labeled-nucleotide dependent. The modified protocols of recovering digoxigenin-DNA was also applied in this biotin-system [14,41,45]. Its use, again, improved the recovery of DNA from 16% to 40%, which is the theoretical maximum labeling efficiency that has been reported for nick translation [5]. This suggested that the molecular chracteristics of biotin-DNA and digoxigenin-DNA may have some similarities. Using Chemiprobe^{TM}

protocols, we had satisfactory recovery of sulfonated DNA.

Selection of Solid Support

The solid phase matrix of filter hybridization serves to separate the sample from other assay reagents, and forms a surface on which the reaction can be visualized and quantitated. Selecting the correct matrix can alter the significance of the results. The successful use of a nonradioactive probe is especially membrane dependent [151].

The binding properties (the affinity of the matrix for a particular substance) of the solid phase are usually the first factor to be considered in selecting a solid support. One of the most important factors to consider with a solid support is the signal-to-noise ratio achieved in a given assay. Non-specific reactions caused by binding of the assay reagents to unoccupied sites on the matrix will obscure the clarity of the final signal, and decrease sensitivity. The solid supports also differ in their compatibility with detection techniques. While a standard nitrocellulose membrane is compatible with virtually all types of probes, some types of nylon membranes used in this study cannot be used with chromogenic labels, because they will create high background levels [176]. Finally, the membrane should also be compatible with the reagents involved in the procedure in terms of physical properties and binding characteristics.

Based upon results established in this study and the considerations described above, Zeta-Probe^{TM} nylon membranes and Immoblin-P^{TM} membranes were inappropriate for the particular application of this study. Zeta-Probe^{TM} nylon membranes caused very high background levels because of nonspecific binding. The physical properties of Immoblin-P^{TM} membranes did not suit bacterial colony blot preparation procedures. Among the other membranes used in this study, Nylon-GTG^{TM}

membranes appeared best in our hands and worked with BlueGENE™ and Chemiprobe™ systems. Nytran™ nylon membranes were the one of choice for the Genius™ and the Chemiprobe™ systems. However, Whatman #541 filter membrane did show potential in the Chemiprobe™ system. It provided a less expensive alternative for either nylon or nitrocellulose membranes. Further efforts at more efficient membrane blocking [151], such as increasing the incubation time and the concentration of blocking reagents, may remove trace nonspecific binding. When both nylon membrane and nitrocellulose membranes work in a system, nylon membrane is usually the preferred choice because of the properties of greater mechanical strength, higher capacity for nucleic acid, stronger retention of bound nucleic acids, and the ability to perform multiple reprobing [9,41,45,136]. Using these probes, the sensitivity results obtained with nylon membranes were equivalent to those obtained with nitrocellulose membranes.

Bacterial Colony Blotting

The protocols for colony blotting on nitrocellulose and nylon membranes used in this study were mainly modified from published work of Grunstein and Hogness [49], Maas [91], Haas and Fleming [50], Kincaid et al. [76], Medon et al. [106], and manufacturer's instruction. The protocols had the following advantages: (1) simplified procedure; (2) no special device required, membranes were simply transferred to absorbent paper saturated with reagent and blotted on filter paper to remove the excess fluid before processing to the next step; (3) no organic solvent used, which made the techniques more convenient to use and nylon membrane became applicable in colony blotting; (4) allowed the use of nonradioactive probes in colony hybridization. The protocols gave

strong signals at positively reacting sites and acceptably low background color.

It was also found in this study that the size of colonies were important for obtaining dense and clear positive color reactions [50]. The lysis of oversized colonies resulted in a smeared colony pattern which made the reading difficult. Colony diameters of 1-2 mm were ideal. Also, incubating the plates at 4° C for 30 minutes before lysing the cells was critical. This step made the colonies stick to the membrane better and decreased smearing. Better results were obtained by inoculating colonies directly on membrane rather than by blotting the membrane. The reason for this is not known.

Proteinase K Treatment For Bacterial Colony Blot

Bialkowska-Hobrzanska has reported [11] that biotin-DNA probes were highly specific when used in protein- and RNA-free DNA preparations in a dot blot hybridization assay. Kincaid and Nightingale [76] demonstrated proteinase K was the best choice among several proteases tested to eliminate the background problem in biotin-based hybridizations. Wetherall et al. [176] and Singer et al. [155] have also reported that treatment with sufficient proteinase K could be expected to cause significant loss of both RNA and protein. In this study, our results further suggested that proteinase K treatment of the membrane was required for bacterial colony blots not only with biotin-DNA probes (BlueGENE™) but also with digoxigenin-DNA probes (Genius™). Though it was not examined in our study, the literature suggests [59,176] that non-specific reactions between sulfonated DNA (Chemiprobe™) and cell components do not occur. Using a radiolabeled probe we found that treatment of bacterial colony blots with proteinase K was not necessary. One of the

advantages of radioactive systems is that we obtain the signals of reactions from reporter-molecules (^{32}P) bound directly to target DNA. This prevents the undesirable reactions between cell components and addition of other reagents (e.g. enzymes, antibody, chromogenic substrate, etc.)

Parameters of Hybridization Reaction

In designing a hybridization experiment, some factors affecting nucleic acid hybridization can be conveniently altered to optimize the method. The temperature of the hybridization and washes and the salt concentration during the washes are the simplest conditions to adjust [4,93]. The stringency increases when the hybridization temperature increases and salt concentration decreases. The high temperature, low salt concentration, and formamide make the formation of hydrogen bonds between two single-stranded DNA less unlikely to happen. For bacterial colony blot hybridization, conditions of high stringency are preferred [41]. All prehybridization and hybridization reactions in this study, therefore, were performed in 45-50% formamide solution. The wash steps using solution III were performed at 60-65° C instead of 50° C. We found that these conditions gave a strong enough positive signal without false positives.

It was also found in this study that the addition of 5% dextran sulfate and 0.5% SDS in both the prehybridization and hybridization solution (but not posthybridization [45]) helped decrease nonspecific binding on unoccupied sites of Nylon-GTG™ membrane in the BlueGENE™ system [9]. The addition of dextran sulfate and SDS help remove the charges on the membrane. It may also be necessary to add dextran sulfate and SDS in pre- and hybridization solution for improving the performance of other kinds of nylon membranes with other nonradioactive DNA detection systems.

Use of Shiga-Like Toxin Gene Probe to Identify EHEC

It has been difficult to undertake studies of the epidemiology of EHEC infections, other than outbreak investigations, because of the lack of suitable methods available to screen large numbers of stool cultures for O157:H7 strains and because of the lack of the knowledge regarding what other serotypes may also be enterohemorrhagic and how to identify them as well [85]. Many efforts have led to the development of sensitive and specific DNA probes to identify EHEC. Levine et al. [85] have described a 3.4 kb DNA probe derived from a 90 kb plasmid present in O157:H7 serotype strains which encodes a fimbrial antigen promoting bacterial attachment of epithelial cells. The probe was very accurate (99%) when used to detect O157:H7 serotype strains; however, it was less accurate for detection of SLT-positive O26:H11 serotype strains (77%) or detection of SLT-positive strains not belonging to either of these serotypes. In addition, some investigators [66,81] have also noted the spontaneous loss of the big plasmid during successive culturing of certain *E. coli* serotype O157:H7 strains. Although all outbreaks of hemorrhagic colitis reported to date have been caused by O157:H7, other serotypes of Verotoxin-producing *E. coli* (VTEC) have been found that occur more sporadically [72]. Krishnan et al. have found three strains of serotype O1 and one untypable strain associated with hemorrhagic colitis [81]. Because SLT synthesis has been strongly implicated as a factor contributing to the pathogenesis of EHEC [122], the DNA probes, designed by Newland and Neill [116], specific for identifying and distinguishing those strains which encode these Shiga toxin-related cytotoxins should be more useful.

The results of this study demonstrated consistent finding for different DNA detection systems. Accuracy of detecting SLT production of O157:H7 strains was 100% (40/40). It also detected a serotype O26 strain

(test No.23), which is now believed to belong to a category of EHEC, giving positive reactions with SLT-I and SLT-II probes. Two VTEC strains (test no.3 & 43), not serotypes O157:H7 or O26, showed positive reaction to SLT-I probe. The results of radioactive and nonradioactive SLT DNA-probe hybridization consistently provided 100% sensitivity (43/43) and 100% specificity (31/31) compared to Verotoxin assay.

Comparison of Different Nonradioactive DNA Detection Systems

Three kinds of nonradioactive labeled-DNA probes and DNA detection systems, biotin-DNA (BlueGENE™ system of BRL), digoxigenin-DNA (Genius™ system of BM), and sulfone-DNA (Chemiprobe™ system of FMC), were used in this study. All share some similarity: 1) Each system can work on nylon membranes, though types of nylon membrane may vary; 2) Minimal concentrations of DNA probes are required in hybridization solution for achieving the required sensitivity -- in this particular application all are between 100 ng/ml to 200 ng/ml; and 3) Labeling efficiencies of DNA probes are very close, 30-40%.

However, there are some advantages and disadvantages of each system over others (Table 8). Chemiprobe™ system benefits by: 1) providing the highest sensitivity of detecting target DNA in the shortest time -- color reactions developed in 30 minutes allow the detection of 0.5 pg of DNA and the best reading can be achieved in one hour; 2) extremely easy procedure of preparing sulfonated DNA probes -- simply add sodium bisulfite and methoxyamine to the denatured DNA, and let the reaction proceed overnight; 3) removing the modification chemical is not necessary -- if the probes are intended to be used in one month, there will be no need to ethanol precipitate the modified DNA, which decreases the chance of DNA loss; and 4) there is no need of proteinase K treatment prior to

prehybridization -- this advantage simplifies the procedure and shortens the probing time. However, the sulfonated DNA is sensitive to reboiling [41] (manufacture indicated reuse of probe only 1-2 times), this might be a disadvantage. One of the advantages of BlueGENE™ is that the biotinylated DNA probes are prepared by nick translation, which can be done in 90 min at 15° C or even in 45 min at 37° C [5,95,132]. This allows hybridization reactions to be performed the same day. The maximal sensitivity achieved in this system is 1 pg after one hour of color development (longer incubation only caused background to develop), which is less sensitive when compared to 0.5 pg with the Chemiprobe™ and Genius™ systems. But if testing time and stability of probes are the important concerns, BlueGENE™ is suitable. Biotinylated DNA probes, in our hands, were reused six times and the performance was consistent. The maximal sensitivity with Genius™ achieved was 0.5 pg after 16 hours of color development. A sensitivity of 0.1 pg claimed by manufacture was never achieved. Moreover, random-prime labeling for digoxigenin-DNA had to proceed overnight (16-18 hours) rather than just one hour in order to get efficient labeling. There was no special advantage of this system over the other two systems in this study.

Comparison Between Nonradioactive and Radioactive DNA Detection Systems

DNA probes are proving to be of increasing value in the clinical laboratory. Conventional, probes are labeled with ^{32}P or ^{35}S. However, these have the inherent problems of radiation exposure, isotope disposal, a requirement for special equipped laboratories, and expense. Most importantly, the practical lifetime of ^{32}P-labeled probes (which were more often used) are limited, and probes must be prepared and standardized

frequently. The nonradioactive probes have advantages over radioactive probes by providing highly stable labeled-probes (stable for at least 1 year if stored at -20° C). These nonradioactive probes are also comparably sensitive and specific, economic, safe and convenient alternatives to the radio-labeled probes (Table 8) [166,176].

It has been observed in this study that efficient hybridization with nonradioactive DNA probes required higher probe concentrations than with ^{32}P-labeled probes. DNA, nonradioactively labeled, would make 2-4 ml of hybridization solution from 1 μg of DNA, whereas DNA radioactively labeled would make 6-18 ml of hybridization solution from 1 μg of DNA. However, one should consider the sensitivity of radioactive probes will decrease with time and be gone very quickly (half-life of ^{32}P is 14.3 days). Furthermore, autoradiography usually takes one to three days exposure in order to get satisfactory positive signals. A similar level of sensitivity can be achieved in one to three hours in nonradioactive systems. The other advantage of the nonradioactive labeling is that the color reactions are visualized directly, so one can stop the reaction whenever the desired sensitivity has been achieved. The radioactive system requires time consuming development of the film.

In this study, the bacterial strains were tested in a blind fashion. The results of the bacterial colony screening with SLT-I and SLT-II probes by both nonradioactive and radioactive DNA detection systems demonstrated complete agreement. They are also consistent with CDC Verotoxin assay confirmation. Both the sensitivity and specificity of SLT-DNA detection with each system, obtained in this study, is 100% (43/43 and 31/31) as compared to verotoxin assay. These results convinced us that nonradioactive DNA detection systems are certainly applicable in our special interest and that the accurate screening of large numbers of bacterial strains is possible.

Table 8. Comparison of One Radioactive and Three Nonradioactive DNA Detection Systems

	BlueGene™	Genius™	Chemiprobe™	^{32}P-DNA
DNA-labeling reaction	Nick translation	Random priming	Sulfonation	Random priming
Labeled-nucleotide	Biotin-dATP	Digoxigenin-dUTP	4-methoxy-5,6-dihydrocytidine-sulfonate	^{32}P-dATP
Conjugate	Streptoavidin-AP	Antibody-AP	Antibody-AP	-
Target DNA detection	Color reaction	Color reaction	Color reaction	Auto-radiography
Use of nylon membrane	Yes	Yes	Yes	Yes
Proteinase K treatment	Yes	Yes	No	No
Sensitivity	1 pg	0.5 pg	≤0.5 pg	≤1 pg
Special equipments required	No	No	No	Yes
stability[a] of Probe	≥1 yr	≥1 yr	≥1 yr	14 days
Reuse of probes	Yes	Yes	Yes[b]	Yes
Cost per sample[c] (dollar)	0.03	0.04	0.07	1.25

AP: Alkaline phosphatase.

a: If stored at -20° C.

b: No more than two times.

c: Calculation were based on resuing the biotin-DNA 6X, digoxigenin-DNA 4X, sulfone-DNA 2X. Cost of ^{32}P-DNA may lower, if label large amount of DNA at a time and reuse the probes within 14 days.

APPENDICES

Appendix I: Transformation of *E. coli* HB101 by pUC19

The transformation procedure were performed according to manufacturer's instructions: 20 µl competent cells was aliquoted into chilled sterile Eppendorf tubes; 3 µl pUC19 was then added to the cells by moving tip through the cells; the mixture was shaken gently for 5 minutes followed by incubating on ice for 30 minutes. After incubation, cells were heat-shocked for 45 seconds in a 42° C water bath and placed on ice for 2 minutes. Room temperature S.O.C. (0.9 ml) was added, and the mixture was shaken at 225 rpm (37° C) for 1 hour. The transformants were spread on LB antibiotic (ampicillin 50 µg/ml) plates for growing and were screened for plasmids by the procedure of Kado and Liu.

S.O.C. (100 ml)	
Bactotryptone	2 g
Yeast extract	0.5 g
NaCl	1 ml 1M NaCl
KCl	0.25 ml 1M KCl
$MgCl_2$, $MgSO_4$	1 ml 2M Mg stock
Glucose	1 ml 2M glucose
Distilled water	97 ml

Bactotryptone, yeast extract, NaCl, and KCl were added to 97 ml of distilled H_2O, allowed to dissolve and then autoclaved. The medium was cooled to room temperature, and 1 ml 2M Mg stock and 1 ml 2M glucose were added. The complete medium was then filtered through a 0.45 µm filter unit to sterilize.

Appendix II: Media

LB (Luria-Bertani) medium (1 liter)	
Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	10 g

Adjust pH to 7.5 with sodium hydroxide. For agar plate medium, before autoclaving, add 15 g bacto-agar. After autoclaving, allow the media to cool by placing in a 50° C water bath. Add 25 mg/ml ampicillin solution to make final concentration as 50 μg/ml.

Appendix III: Reagent Preparation for Plasmid Isolation

3 M Ammonium Acetate	100 ml
ammonium acetate	23.12 gm

Dissolve in 50 ml deionized water, adjust pH to 4.8 with glacial acetic acid, bring up to 100 ml with deionized water, autoclave 15 min at 121° C.

Lysing Solution	100 ml
SDS	3 gm
Tris	12.11 gm

Dissolve in 100 ml deionized water, adjust pH to 12.6 with 5-6 HaOH pellets. The pH of this solution is very critical. If the pH is too high, it will also denature and destroy plasmid DNA.

TE Buffer	1000 ml
Tris-HCl	1.58 gm
EDTA	0.34 gm

Adjust the pH to 8.0 with NaOH, sterilize at 121° C for 15 min.

TE Saturated Phenol-Chloroform	
Phenol	130 ml
Chloroform	130 ml
Isoamyl alcohol	5.5 ml

TE buffer	300 ml

Bring the phenol (-20° C, crystilline form) to room temperature, then melt it in a 65° C water bath. Remove the phenol from the water bath as soon as it has melted (about 130 ml liquid).

Appendix IV: Purification of P32-labeled DNA Through NACS PREPAC Mini-Column

NACS is an ion exchange resin that can bind nucleic acids in low salt (0.1-0.5 M NaCl) and release them in high salt (0.7-2.0 M NaCl). The procedure of removal of unincoporated nucleotide from labeled polynucleotides is divided into three steps: (1) wash the resin with high salt buffer and then with the specified low salt buffer, (2) load nucleic acid solution on to the resin in the same specified low salt buffer and continue to wash with low salt buffer to remove unbound material, (3) elute nucleic acid with the specified high salt buffer.

1. Wash the resin 3 times, 1 ml each time, with 2.0 M NaCl in TE buffer (10mM Tris-HCl, pH 7.2 1 mM EDTA). This is accomplished by attaching the PREPAC to the barrel of a 1-ml Pipetman. The solution is drawn up through the bottom of the column into the reservoir and then expelled by depressing the plunger.

2. Wash the resin with low salt buffer 0.5 M NaCl in TE for SLT-I and 0.2 M NaCl in TE for SLT-II. The physical technique for equilibrium is the same as that used in step 1.

3. Remove pipetman and clamp the PREPAC on to a ring stand. The sample is added to the top of the column and allowed to flow through by gravity flow in order to maximize binding. Add the same buffer indicated at step 2 to the column reservoir attached to the top of mini-column.

4. Collect the elution 0.8 ml each tube, check radioactivity with each of them, and stop low salt buffer washing when the radioactivity are gone.

5. Elute the PREPAC three times with 100-200 µl of high salt buffer (2.0 M NaCl in TE buffer for SLT-I and 1.0 M NaCl in TE buffer for SLT-II). Combine the two tubes which have highest activity.

Appendix V: Prehybridization, Hybridization, & Posthybridization Solution

Prehybridization Solution 10 ml

For BlueGENE™ and ^{32}P-labeled probing systems:

Formamide	5 ml
20X SSC	2.5 ml
Denhardt's solution	1 ml
1 M NaH_2PO_4	0.25 ml
Dist. water	0.75 ml
10 mg/ml Freshly denatured herring sperm DNA	0.5 ml

— 20X SSC (20X standardized saline citrate): dissolve 173 gm NaCl and 88.2 gm sodium citrate in 800 ml dist. water. Adjust pH to 7.0 with 5 N NaOH and adjust volume to 1000 ml. Sterilize by filtering.

— To denature the sperm DNA, boil the DNA at 95°C for 10 minutes and then quickly chill on ice.

— When using nylon membranes with nonradioactive system, add 5% dextran sulfate and 0.5% SDS to the prehybridization solution.

For Genius™ probing system:

Formamide	5 ml
20X SSC	2.5 ml
Blocking reagent	0.5 gm

10% Lauroylsarcosine	0.1 ml
5% SDS	0.4 ml
Dist. water	2 ml

For Chemiprobe™ probing system:

Formamide	5 ml
SDS	0.1 gm
NaCl	0.58 gm
Dextran sulfate	0.5 gm
Dist. water	4.9 ml
10 mg/ml freshly denatured herring sperm DNA	0.1 ml

Hybridization Solution 10 ml

For BlueGENE™ and ^{32}P-labeled probing systems:

Formamide	4.5 ml
20X SSC	2.5 ml
dextran sulfate	0.5 gm
Denhardt's solution	0.2 ml
1 M NaH_2PO_4	0.2 ml
Dist. water	2.4 ml
10 mg/ml Freshly denatured sperm DNA	0.2 ml

For Genius™ & Chemiprobe™ systems:

Hybridization solution is same as prehybridization solution.

Posthybridization solution 1000 ml

Solution I (2X SSC/0.1% SDS)	
20X SSC	100 ml
SDS	1 gm
Dist. water	900 ml
Solution II (0.2X SSC/0.1%SDS)	
20X SSC	10 ml
SDS	1 gm
Dist. water	990 ml
Solution III (0.1X SSC/0.1%SDS)	
20X SSC	5 ml
SDS	1 gm
Dist. water	995 ml

Appendix VI: Buffers & Reagents Used in Target DNA Detection Systems

BlueGENE™

* Buffer 1: 2000 ml

Tris-HCl	31.52 gm
NaCl	17.54 gm

Dissolve in 1,500 ml deionized water, adjust pH to 7.5 with 5N NaOH, adjust volume to 2,000 ml.

* Buffer 2: 100 ml

Bovine serum albumin	3 gm
Buffer 1	100 ml

* Buffer 3: 2,000 ml

Tris-HCl	31.52 gm
NaCl	11.70 gm
$MgCl_2.6H_2O$	20.33 gm

Dissolve in 1,500 ml deionized water, adjust pH to 9.5 with 5N NaOH, adjust volume to 2,000 ml.

* Buffer 4: 1,000 ml

Tris-HCl	3.16 gm
Na_2EDTA	0.168 gm

Dissolve in 500 ml deionized water, adjust pH to 7.5 with 1N NaOH, adjust volume to 1,000 ml. Filter the solution through a 0.45 μm filter units to sterilize.

* Dye solution	(for 100 cm^{-u2} filter)	
Buffer 3	7.5 ml	
NBT	33 μl	gently mixed by inverting the tube
BCIP	25 μl	gently mixed.

Genius™

* Buffer 1,3 & 4 are the same as those of BlueGENE™.

* Buffer 2: 200 ml	
Blocking reagent	4 gm
Buffer 1	200 ml

* Dye solution	(for 100 cm^2 filter)
Buffer 3	10 ml
NBT	45 μl
BCIP	35 μl

Chemiprobe™

* Wash solution: 1,000 ml	
NaCl	30 gm
30% Brij 35 nonionic detergent	3 ml

* Phosphate buffered saline: 500 ml

NaCl	2.925 gm
Tris-HCl	1.576 gm
EDTA	0.186 gm

Dissolve in 400 ml deionized water, adjust pH to 7.4-7.5, adjust volume to 500 ml.

* Blocking solution I (with heparin):	for 100 cm^2 filter	
2X blocking solution diluent	1.25 ml	
10,000 units/ml heparin	0.125 ml	
Dist. water	1.125 ml	mixed well
Blocking powder	0.75 gm	

* Blocking solution II (without heparin):	for 100 cm^2 filter	
2X blocking solution diluent	1.25 ml	
Dist. water	1.25 ml	mixed well
Blocking powder	0.75 gm	

* Dye solution: 5 ml

Substrate buffer	5 ml	
NBT	15 mg	mixed well
BCIP	20 µl	

Store at -20° C for 1-2 weeks.

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