DETECTION OF *Escherichia coli* IN WATER AND FOOD BY USING DNA PROBE AND POLYMERASE CHAIN REACTION

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THESIS
entitled

DETECTION OF *ESCHERICHIA COLI* IN WATER AND FOOD BY USING DNA PROBE AND POLYMERASE CHAIN REACTION

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ชื่อวิทยานิพนธ์	การตรวจสอบการปนเปื้อนของ เชื้อ *Escherichia coli* ในน้ำและอาหาร โดยการใช้ดีเอ็นเอตรวจสอบและเทคนิค polymerase chain reaction
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บทคัดย่อ

การวิจัยนี้ได้นำดีเอ็นเอโพรบและวิธีการทำ PCR มาใช้ เพื่อพัฒนาวิธีการตรวจสอบเชื้อ *Escherichia coli* ในน้ำและอาหาร ดีเอ็นเอโพรบ 4 ขนาดที่ใช้เป็นส่วนของยีน *uidA* ของ *E. coli* K12 ซึ่งสร้างเอนไซม์ β-glucuronidase (GUS) เมื่อติดฉลากดีเอ็นเอโพรบเหล่านี้ด้วย digoxigenin และนำไปทดสอบกับแบคทีเรียต่าง ๆ โดยใช้เทคนิค dot blot hybridization ผลการทำไฮบริไดเซชันพบว่า ดีเอ็นเอโพรบทั้ง 4 สามารถไฮบริไดซ์กับ *E. coli* ที่นำมาทดสอบทั้งหมด 120 สายพันธุ์ โดยไม่ขึ้นอยู่กับ การสร้างเอนไซม์ GUS และให้ผลลบ กับเอ็นเตอริคแบคทีเรียตัวอื่นๆ คือ *Citrobacter, Enterobacter, Edwardsiella, Morganella, Klebsiella, Proteus* และ *Salmonella* รวมทั้งแบคทีเรียแกรมบวก ที่ใช้ในการทดสอบคือ *Bacillus* และ *Staphylococcus* แต่ให้ผลบวกกับ *Shigella* จำนวน 11 สายพันธุ์ทั้งที่สร้าง และไม่สร้างเอนไซม์ GUS การใช้เทคนิค PCR โดยใช้ primer 2 คู่ ที่ได้จากส่วนของยีน *uidA* คือ GAL-301 กับ GAR-432 ซึ่งจะให้ product ขนาด 0.153 kb และ GAL-301 กับ GAR-806 ซึ่งจะให้ product ขนาด 0.527 kb เป็นการทดสอบเพื่อยืนยันว่า primer จากส่วนของยีนดังกล่าวมีความจำเพาะสูงในการตรวจสอบเชื้อ *E. coli* จากผลการทดสอบ ความจำเพาะแสดงให้เห็นว่า ดีเอ็นเอโพรบทั้ง 4 คือ GA153, GA527, GA175 และ GA549 และ primer ทั้ง 3 คือ GAL-301, GAR-432 และ GAR-806 มีความจำเพาะสูงในการใช้ตรวจสอบ *E. coli* และ *Shigella* ทุกสายพันธุ์ทั้งที่สร้าง และไม่สร้างเอนไซม์ GUS การทดสอบความไวของวิธีการพบว่า เทคนิค dot blot hybridization โดยใช้ดีเอ็นเอตรวจสอบติดฉลากด้วย digoxigenin มีความไวในการตรวจสอบ เชื้อได้ประมาณ

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10^5 CFU/dot ขณะที่ เทคนิค PCR จะสามารถตรวจพบเชื้อได้ถ้ามีเชื้อ 1-10 เซลล์ต่อหนึ่งปฏิกิริยา PCR และตรวจสอบผลด้วยวิธีเจลอิเลคโตรโฟเรซิส ได้ทำการตรวจสอบ *E. coli* ในน้ำโดยใช้เทคนิค PCR ร่วมกับเทคนิคการกรองน้ำด้วย FHLP membrane (Millipore Corp.) พบว่าตรวจไม่พบ *E. coli* ในน้ำดื่ม 10 ตัวอย่างที่ใช้ในการทดสอบ ได้ใช้เทคนิคการทำ PCR โดยตรงและการใช้ PCR ร่วมกับเทคนิคการกรองมาใช้ตรวจสอบ *E. coli* ในตัวอย่างอาหาร จากการทดสอบพบว่า ขั้นตอน preenrichment สามารถเพิ่มจำนวน PCR products ซึ่งแสดงถึงจำนวนเซลล์ที่เพิ่มขึ้นจากการบ่ม อย่างไรก็ตามมีผลการทดลองแสดงให้เห็นว่าการใช้เทคนิค PCR สามารถตรวจพบ DNA อิสระ (1 นาโนกรัม) ซึ่งปนเปื้อนอยู่ในตัวอย่างอาหารได้ แม้ว่าจะผ่าน การบ่มที่ 37°C เป็นเวลา 0, 30 และ 60 นาที แล้วก็ตาม ผลการทดลองดังกล่าวชี้ให้เห็นว่า มีความเป็นไปได้ที่ การใช้เทคนิค PCR จะสามารถตรวจพบ DNA อิสระ หรือ DNA ที่มาจากเซลล์ที่ตายแล้ว ซึ่งปนเปื้อนอยู่ในตัวอย่างที่ใช้ทดสอบได้ ดังนั้นการแปลผลการตรวจสอบแบคทีเรียในน้ำและอาหารด้วยเทคนิค PCR จึงต้องมีปัจจัยควบคุมที่เฉพาะเจาะจง

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Thesis Title Detection of *Escherichia coli* in Water and Food by using DNA probe and Polymerase Chain Reaction.

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ABSTRACT

To develop the alternative method for detection of *Escherichia coli* in water and food, specific DNA probes and polymerase chain reaction methods for monitoring the presence of this bacteria were studied. The four digoxigenin labeled DNA probes derived from *uidA* gene of *E. coli* K12 which encodes β-glucuronidase (GUS) namely GA153, GA527, GA175, and GA549 were used in dot blot hybridization with bacterial cultures. These four probes hybridized and gave positive signal to all 120 strains of *E. coli* tested in this study independent of GUS phenotype. Other enteric bacteria except *Shigella* such as *Citrobacter, Enterobacter, Edwardsiella, Morganella, Klebsiella, Proteus* and *Salmonella* and other gram positive bacteria including *Bacillus* and *Staphylococcus* were not reactive with these probes. All 11 strains of *Shigella* which are both GUS positive and negative could hybridize well with the four probes. PCR amplification with two specific sets of primers derived from

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uidA gene, GAL-301 with GAR-432 and GAL-301 with GAR-806 which gave product of 0.153 kb and 0.527 kb, respectively also confirmed the high specificity of detection of *E. coli* using primers from such gene. The observed data demonstrated that the use of either one of the four probes, GA153, GA527, GA175 and GA549, or the 2 pairs of the three primers, GAL-301, GAR-432 and GAL-806 proven to be highly specific for detection of *E. coli* and *Shigella* which may or may not have GUS activity. The dot blot hybridization technique, using digoxigenin labeled probes could detect *E. coli* at 10^5 CFU/dot and the sensitivity could be brought down to detect only 1 to 10 bacteria per reaction if the PCR technique was used. Detection of *E. coli* in water sample could be done using PCR amplification together with filtration technique with FHLP membrane (Millipore Corp.). The detection of *E. coli* in 10 brands of drinking water was performed by such combined technique. The result showed that *E. coli* was not found in all the ten brands of drinking water samples. Direct PCR amplification and PCR combined with filtration method to detect *E. coli* in food sample were tested. The preenrichment step increased the yield of PCR products which indicated the increase of bacterial cells upon incubation. However there was evidence demonstrated that PCR amplification could detect DNA (1 ng) which was contaminated in food sample even after incubation at 37°C for 0, 30 and 60 min. Such finding indicated that if the sample contaminated with nonviable cells or intact DNA, there was possibility that PCR technique will be able to detect DNA released from those cells or free DNA that was present in the sample. Therefore, it is suggested that the interpretation of bacteria detection in water or food by PCR technique samples requires specific monitoring factors.

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LIST OF ABBREVIATIONS

bp	base pair
°C	degree celcius
CFU	colony forming unit
EAggEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
et al.	Et. alii (latin), and others
Fig.	figure
GUS	β-glucuronidase
H	flagella antigen
hr	hour
K	capsular antigen
kb	kilobase
LT	heat-labile
μg	microgram
μl	microlitre
μm	micrometre
μM	micromolar
min	minute

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(continued)

ml	millilitre
mm	millimetre
mM	millimolar
M	Molar
MPN	Most probable number
MU	methylumbelliferone
MUG	methylumbelliferyl-β-D-glucuronide
ng	nanogram
nM	nanometre
O	somatic antigen
PCR	Polymerase chain reaction
s	second
spp.	species
ST	heat-stable
UV	ultraviolet

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CHAPTER I

INTRODUCTION

The occurrence of fecal coliforms in foods and water is regarded as an important indication of public health hazard from infectious agents. Coliforms, fecal streptococci, *Escherichia coli*, and enteropathogenic, enterotoxigenic *E. coli* have all been used as sanitary indicators of fecal pollution in foods and water (Chordash et al., 1978). *E. coli* is often preferred over the coliform group as an indicator because it is specific and most reliably reflects fecal origin (Feng et al., 1982). Current conventional assays for *E. coli* are based on the properties of acid or gas production from lactose. Some of the accepted methods include the Most-Probable-Number (MPN) method. All these tests are laborious and time consuming, some require 96 hours to complete (Feng et al., 1982). So the more rapid and sensitive methods have been developed by using of microbial enzyme profiles to detect indicator bacteria. A fluorogenic assay for the rapid identification of *E. coli* in food and water is based on the presence of the enzyme β-D-glucuronidase (GUS) which cleaves the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) to release a fluorogenic radical, methylumbelliferone (MU) (Feng et al., 1982). When the MUG compound is incorporated into conventional bacteriological media, the presence of *E. coli* can be easily determined by the bluish fluorescence in the medium that is observable under long-wave UV light (Framton et al., 1993). However, about 34% of the human fecal isolates of *E. coli* examined showed a negative reaction to the MUG test (Chang et al., 1989). There is also other reports indicates that about 94-97% of *E. coli* contains

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GUS activity (Kilian et al., 1976, 1979). In addition, some *E. coli* strains such as the pathogenic enterohemorrhagic strains lack GUS activity (Framton et al., 1993, Willshaw et al., 1994). So the result of this GUS detection procedure may not be entirely satisfactory. Therefore an alternative approach as the method for *E. coli* detection attracts attention to be studied.

To achieve the efficient and facile method for water and food monitoring purpose, new technologies, such as DNA probe and Polymerase Chain Reaction (PCR) techniques have been developed. Though, not all *E. coli* strains are GUS positive, there was report that GUS negative *E. coli* strains still harbor genes for GUS (*uid* operon), but somehow the genes are not expressed (Feng et al., 1991). The objectives of this study are to check the possible use of DNA probe and PCR technology to detect *E. coli* present in water and food samples. In this study, the region of *uidA* gene of *E. coli* which coded for enzyme GUS was selected to serve as the target for *E. coli* detection by both methods. DNA fragments which contained the part of *uidA* gene were used as DNA probe to analyzed for specificity and sensitivity for *E. coli* detection by dot blot hybridization. PCR was also performed by using primers based on the *uidA* gene . To check the specificity of the methods, gene probe and PCR amplification detection were tested with several *Enterobacteriaceae* and some gram positive bacteria. Some water and food samples were also tested by these methods. Food samples mixed with either *E. coli* or its chromosome were also subjected to PCR amplification to check for its ability for *E. coli* detection in the presence of food materials.

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Chapter II

Background

1. Morphological and cultural characters of *Escherichia coli.*

Eschrichia coli originally was isolated and was designated under different name. It was named and described as *Bakterium coli* in 1885 by Escherich who isolated an organism from the faeces of infants. The name '*Bacterium coli*' was subsequently widely used for many years. Until, in 1920 the name of this bacteria was changed to *Escherichia* in honor of its discovery (Holmes et al., 1990, Brock et al., 1991). *Escherichia coli* is the member of the family *Enterobacteriaceae* which is in section 5 of Bergey's manual, a widely accepted reference for identification of bacteria. It is the best studied bacterium and the choice of experimental organism for many microbiologists. Some strains are important members of the normal gut flora in man and animals whereas others possess virulence factors which enable them to cause infections in the intestinal tract or at other sites, particularly the urinary tract (Mims et al., 1993). *E. coli* is defined as facultatively anaerobic, gram-negative, non-sporing, rod-shaped bacteria. Some strains of *E. coli* are motile by peritrichous flagella but some strains are nonmotile. Some other strains, especially those from extra-intestinal infections, posses a polysaccharide capsules or microcapsules (Orskov F., 1984). This bacteria grows over a wide range of temperature (15-45°C). There was report that some strains are more heat resistant than other members of *Enterobacteriaceae* and will survive treatment with 60°C for 15 min or 55°C for 60 min (Holmes et al.,

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1990). The appearance of colonies on nutrient agar may be smooth (S) , low convex, moist, gray, with shiny surface and entire edge and easily dispersible in saline, or they may be rough (R), dry and difficult to disperse well in saline. Upon growing on MacConkey's agar, they form large red colonies and on blood agar, around the colony growth is discolored and there may be hemolysis. In addition to the proteinaceous flagella, most strains have fimbriae (pili) or fibrillar proteins often extending in great numbers from the bacterial surface and far out into the surround medium. They have a width of 5-9 nm (Duguid J.P., 1959, and Brinton C.C., 1965). Some fimbriae have another specific functions as adhesieve organs. In some cases mucoid and slime-producing forms occur. Oxidase test is negative. The growth of *E. coli* on carbon source shows that, acetate can usually be used as a sole carbon source, but citrate cannot be used. Glucose and other carbohydrates are fermented with the production of pyruvate, which is further converted into lactic, acetic and formic acids. Part of the formic acid is splitted further by a complex hydrogenlyase system into equal amounts of CO_2 and H_2. Particular strains of *E. coli* are anaerogenic. Lactose is fermented by most strains but fermentation may be delayed or absent. The mol% G+C of the DNA is 48-52 (Orskov F, 1984). *E. coli* is genetically very related to *Shigella*. The four species of *Shigella* which *are S. boydii, S. dysenteriae, S. frexneri,* and *S. sonnei* and *E. coli* can be identified as a single species on the basis of DNA relatedness (Brenner et al., 1972, 1973). In fact they are so similar that they are able to undergo genetic recombination with each other and are susceptible to some of the same bacteriophages. Tests for DNA homology show strains of *Shigella* having 70 to nearly 100% homology with *E. coli* (Brock et al., 1991). *E. coli* is the most abandant bacteria.

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The alimentary tract of most warm-blooded animals typically is colonized within a few hours or days of birth by *E. coli* from ingested food or water or directly from other individuals. Most *E. coli* strains can adhere to the mucus overlying the surface of the large bowel and distal small bowel. It has been estimated that the doubling time of *E. coli* within the intestine is about 40 hours (Falkow et al., 1990). Once an *E. coli* strain becomes established within a host, it may persist for months or years. Usually other *E. coli* strains are constantly introduced, but most persist for only a few days or weeks. Resident strain(s) usually change over a long period, but much more rapidly after enteric infection or antimicrobial therapy. *E. coli* of the normal flora provides protection against colonization by harmful microorganisms (Falkow et al., 1990).

2. *E. coli* diversity.

Subdivision of *E. coli* can be carried out in many ways such as phage typing, colicin typing, biotyping, typing by outer membrane protein (OMP) pattern, typing by antibiotic resistance patterns and typing by direct hemagglutination, but serology is one of the most useful ways to subdivide the species on a global basis (Orskov F., 1984). This method is based on the many antigenic differences found in structures on the bacterial surface. The main aspect of this analysis is the O antigen determination based on antigenicity of the lipopolysaccharide (LPS); 171 O antigens are presently listed, many of which cross-react to each other (Orskov F., 1984).

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The K antigens, which originally were defined exclusively according to their agglutination abilities, have been redefined and now the definition is also based on their

chemical nature. The K antigens are the polysaccharide capsular antigens. Nearly 80 different K antigens are known (Orskov F., 1984).

Fragellar or H antigens make up the third main group of serotyping antigens. A total of 56 H antigens are established. A serovar is recorded in the following way: O18acK1:H7 or O111:H2. The latter antigenic formula indicates that K antigens are not present in the strain (Orskov F., 1984).

E. coli O, K, and H antigens are usually determined by chromosomal genes. Although the serotypes do not necessarily provide an index of overall genetic similarity among strains, the correlation with pathogenicity is high, and serotyping has been of extraordinary importance in distinguishing the small number of strains that actually cause disease (Falkow et al., 1990). Table 1 show some O groups, O:H, and O:K:H serovars from human *E. coli* enteropathies.

Table 1 Some O Groups, O:H, and O:K:H serovars from human *E. coli* enteropathies.

Infantile Diarrhea EPEC[a]	Diarrhea in Adults and Children	
	ETEC[b]	EIEC[c]
026, 044, 055, 086, 0111, 0114, 0119, 0125, 0126, 0127, 0128, 0142, 0158	O6:K15:H16, O8:K40:H9, O8:K47:H-, O8:K25:H9, O11:H27, O15:H11, O20:H-, O25:K7:H42, O25:K98: H-; O27:H7, O27:H20, O63:H12, O73:H45, O85:H7; O78:H11, O78:H12, O114:H21; O115:[H51],[d] O128:H7, O128:H12, O128:H21, O139:H28, O148:H28, O149:H4, O159:H4, O159:H20, O159:H34; O166:H27, O169:H-	028ac, 0112, 0124, 0136, 0143, 0144, 0152, 0164

[a] EPEC, enteropathogenic *E. coli.* The O groups are listed; however, only a limited number of O:H types have been shown to have an association with infantile diarrhea.
[b] ETEC, enterotoxigenic *E. coli.* The data presented are primarily from Ørskov and Ørskov (1980). 0166 = OX8, and 0169 = OX2.
[c] EIEC, enteroinvasive *E. coli.*
[d] [ ], nonmotile variants exist.

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(From Orskov F., 1984)

3. Pathogenesis and pathogenic *E. coli* strains

Specific strains of *E. coli* express the pathogenic traits. The three principal kinds of disease are urinary tract infections, neonatal meningitis and intestinal (diarrheal) diseases. Mechanistically, these diseases depend on combinations of bacterial properties : adherence to specific host receptors, elaboration of specific exotoxins, and penetration (invasion) of host cells (Falkow et al., 1990).

3.1 Urinary tract infection.

For urinary tract infection, 90% of infections are caused by *E. coli*. The ability to cause infection of the urinary tract is limited to certain serogroups of *E. coli* (e.g. O1, O2, O4, O6, O7 and O75 ; these serogroups differ from those associated with gastrointestinal tract infection.). The success of these strains may be attributable in part to their ability to colonize the periurethal areas (Mims et al., 1993). The uropathogenic strains are present in the stool and subsequently colonize the vaginal and periurethal region. Infection is usually acquired by the ascending route from the urethra to the bladder and may proceed to the kidney (Falkow et al., 1990). Some *E. coli* possess particular types of fimbriae (pili) which enable them to adhere to urethral and bladder epithelium by specific protein at the tip of pili called adhesins (Mims et al., 1993). Other features of *E. coli* appear to assist in selective localization of organisms in the kidney, provide pathogenesis and lead to renal damage. The capsular acid polysaccharide (K) antigens are among such property which associated with the ability to cause pyelonephritis and are known to enable *E. coli* strains to resist host defences

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by inhibiting phagocytosis. Hemolysin production by these strains may also be linked with the capacity to cause kidney damage (Mims et al., 1993).

3.2 Neonatal meningitis.

The most common bacterial agents for neonatal meningitis is *E. coli* which affecting about 1 in 2,000 to 4,000 infants. Approximately 80% of the isolates synthesize the K1 capsular polysaccharide. *E. coli* K1 strains are found in the colonic flora of 20% to 40% of all individuals (Falkow et al., 1990). It is most likely that neonates acquire the strains from their mothers through the nasopharynx or by swalling during the process of the passage through birth canel. The organism move to the intestine, after which invading through the blood stream will eventually lead to the meninges (Falkow et al., 1990).

3.3 Intestinal diseases.

As stated above, *E. coli* is a predominant species of normal flora in human intestine. It plays an important role in maintaining intestinal physiology. There are several pathogenic *E. coli* strains that cause distinct syndrome of diarrheal disease (Levine M.M., 1987). There are four well-recognized categories of diarrheagenic *E. coli* based on distinct virulence properties, different interactions with the intestinal mucosa, distinct clinical syndromes, differences in epidemiology, and distinct O:H serotypes. The four main categories of diarrheagenic *E. coli* are enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) (Levine M.M., 1987). Recently, a fifth

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category of *E. coli*, recognizable by its aggregative or "stacked-brick" type of adherence to cultured mammalian cells, has been recognized as yet another category of diarrheagenic *E. coli* in children in different part of the world. Because of its characteristic aggregative type of adherence, this *E. coli* has been referred to as enteroaggregative *E. coli* (Albert et al., 1993).

Although the four main categories of diarrheagenic *E. coli* are quite distinct, they nevertheless have certain underlying commonalities with respect to pathogenesis: 1) critical virulence properties are encoded in plasmids 2) characteristic interaction with intestinal mucosa 3) production of enterotoxins or cytotoxins and 4) in each category tend to fall within certain O:H serotypes (Levine MM., 1987).

3.3.1 Enterotoxigenic *E. coli* (ETEC).

Enterotoxigenic strains are an important cause of diarrhea in infants and travelers in less developed countries (Schultsz et al., 1994). The disease range from minor discomfort to severe cholera-like, purging liters of fluid per day leading to severe dehydration, particularly in children. The ETEC are acquired by the ingestion of contaminated food and water. Adults in endemic areas are evidently usually immune. Ordinarily, large numbers of organisms must by consumed to cause disease in a susceptible individual. The process requires intestinal colonization as well as the elaboration of one or more enterotoxins. Both of these traits are encoded on plasmids (Falkow et al., 1990). ETEC possess attachment or colonization factors that allow them to overcome the peristaltic defense mechanism of the small intestine. CFA-1, CFA-2 and E8775 are examples of fimbrial adhesins or colonization factors found in human ETEC (Levine MM., 1987).

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E. coli that synthesizes only a colonization factor but no toxin may cause a mild diarrheal disease, because their adhesion to the small bowel in large numbers causes a malabsorption syndrome. However, true ETEC strains synthesize one or both of two plasmid-mediated enterotoxins : heat-stable (ST) or heat-labile (LT) (Falkow et al., 1990). Either causes net secretion of fluid and electrolytes into the lumen of the bowel. ETEC strains usually possess two distinct plasmids, one encoding a colonization factor, and another encoding one or two enterotoxins ; but in some strains the LT and ST genes may reside on separate plasmids (Falkow et al., 1990). LT is very similar in structure and mode of action to cholera toxin produced by *Vibrio cholerae* involving in increasing of cAMP, and infections with these strains can mimic cholera, particularly in young and malnourished children (Mim et al., 1993). STs have a similar but distinct mode of action to that of LT. There are two types of heat stable enterotoxins. ST_A activates guanylate cyclase activity causing an increase in cyclic guanosine monophosphate, which results in increased fluid secretion. The other ST toxin is ST_B (or ST Ib). The mechanism of action of ST_B (or ST Ib) is unknown. Unlike LT, ST does not stimulate an immune response in infected individuals so it cannot be detected by immunological tests (Mim et al., 1993). Some ST genes reside on a transposon (Falkow et al., 1990),

3.3.2 Enteropathogenic *E. coli* (EPEC).

Investigators in the 1940s and 1950s applied serotyping system to define the EPEC as strains of O:H antigen types epidemiologically incriminated in nosocomial and community outbreaks of neonatal diarrhea (Donnenberg et al, 1992). EPEC causes disease in the very young, rarely affecting children over 1 year of age and most

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closely associated with diarrhea in those under 6 months (Gomes et al., 1991 and Levine et al., 1984). In addition, EPEC infection is recognized as cause of chronic diarrhea (Bower et al., 1989 and Clausen et al., 1982). Nosocomial neonatal diarrhea due to EPEC, although now rare in developed countries, still occurs in developing nations and still has high mortality (Donnenberg et al., 1992).

Although these strains induce a watery diarrhea not unlike that seen with ETEC strains, EPEC do not produce either LT or ST, nor do they possess the same colonization factors as ETEC. The EPEC cells adhere tightly to the surface of intestinal epithelial cells (in either a diffuse or clustered pattern), indenting the enterocyte membrane and causing localized destruction of the microvilli but without overt invasion of the cells (Falkow et al., 1990). The capacity of EPEC strains to adhere tightly to cultured human cells is frequently associated with a plasmid (Falkow et al., 1990, Baldini et al., 1983, and Nataro et al., 1985a). The precise nature of the EPEC adherence factor (EAF) is not know ; it does not appear to be fimbriae. The genes associated with EAF have been cloned, and their use as a DNA probe showed that they were restricted to serotypes implicated in worldwide EPEC outbreaks (O55, O111, O119, O127, and O128) (Falkow et al., 1990, Baldini et al., 1986, Echeverria et al., 1991, Gomes et al., 1989, Kain et al., 1991, Levine et al., 1988, and Nataro et al., 1985b). EPEC strains have also been found to elaborate a cytotoxin that is enterotoxic and similar to a toxin synthesized by some *Shigella*. Evidently, the pathogenesis of EPEC involves a specialized adhesion followed by synthesis of sufficient cytotoxin to have an enterotoxic effect (Falkow et al., 1990).

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3.3.3 Enteroinvasive *E. coli* (EIEC).

E. coli strains that caused an invasive, dysenteric form of diarrhea illness was described in 1971 by DuPont et al. These strains, of serotypes distinct from ETEC and EPEC, closely resembled *Shigella* in many ways (Levine MM., 1987). Like *Shigella*, their cardinal pathogenetic feature is the capacity to invade and proliferate within epithelial cells and cause eventual death of the cell (Dupont et al., 1971). The large (about 140 Mda) plasmids correlate with the invasive capacity of both EIEC and *Shigella* (Harris et al., 1982). The plasmid codes for the production of several outer membrane proteins involved in invasiveness ; the proteins are antigenically closely related (if not identical) between EIEC and *Shigella* (Hale et al., 1983). Moreover EIEC often resemble *Shigella* character that they are nonmotile and lactose-negative, cross-react with certain *Shigella* O antigens (Levine MM., 1987). It can be said that EIEC penetrate and multiply within epithelial cells of the distal ileum and colon of primates, causing widespread cell destruction. The organisms rarely enter the blood stream in individuals who are adequately nourished. The clinical syndrome is identical to shigellla dysentery, as is its treatment and prevention (Falkow et al., 1990).

3.3.4 Enterohemorrhagic *E. coli* (EHEC).

The term EHEC strains refers to a class of diarrheagenic *E. coli* which carries the genetic determinants for a Haching-effacing lesion and Shiga-like toxin production (SLT) (Begum et al., 1993). EHEC strains are capable of causing diseases including diarrhea, hemorrhagic colitis, and heamolytic uremic syndrome (Paton et al., 1993). EHEC strains belonging to serogroup O157 are most commonly associated with EHEC strains cause a diarrheal syndrome distinct from the bacillary dysentery caused

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by *Shigella* and EIEC. The clinical syndrome is notable in that bloody but copious diarrhea, unaccompanied by fecal leukocytes (Riley et al., 1982) while the classic dysentery due to *Shigella* or EIEC infections are characterized by fever and scanty stools of blood and mucus containing many fecal leukocytes (DuPont et al., 1971). The EHEC strains do not elaborate LT or ST nor invade epithelial cells. However, they possess a 60- to 70- Mda plasmids that is associated with virulence. The EHEC strains also produce one or both principal cytotoxins. One of these toxins, Shiga-like toxin 1 or Verotoxin 1 or VT_1, is apparently identical to the potent cytotoxin /neurotoxin /enterotoxin produced by *S. dysenteriae* type 1 (Shiga toxin) (Brown et al., 1982, and Eiklid et al., 1983) and reacts with and is neutralized by antibody to Shiga toxin. Many strains also elaborate a second potent cytotoxin (Shiga-like toxin 2 or Verotoxin 2 or VT_2) that is not neutralized by antibody to Shiga toxin (O'Brien et al., 1983, Scotland et al., 1985, Strockbine et al., 1986, and Noda et al., 1985). The toxin is encoded by bacteriophages (O'Brien et al., 1984, Smith et al., 1984, and Scotland et al., 1985). Strains of O157 commonly produce VT2 only, strains elaborating both toxins are found less frequently , a VT1 producer are very rare (Willshaw et al., 1994)

3.3.5 Enteroaggregative *E. coli* (EAggEC).

The fifth category of *E. coli* have been defined as etiologic agents of gastroenteritis in infants in developing countries recently (Bhan et al., 1989, Cravioto et al., 1991, Nataro et al., 1987). EAggEC strains are characterized by their distinctive aggregative adherence to Hep-2 or HeLa cells in culture (Nataro et al., 1987, and Vial et al., 1988). The distinct aggregative adherence is also associated

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with the presence of a 60- Mda plasmid that encodes bundle-forming fimbriae 2 to 3 nm in diameter (Nataro et al., 1992). Recently, a plasmid-mediated heat-stable enterotoxin and a heat-labile protein antigenically related to the *E. coli* hemolysin produced by EAggEC have been reported (Baldwin et al., 1992, and Savarino et al., 1991). It has also been reported that EAggEC strains produce hemorrhagic lesions in the rabbit and rat ileal loops, and the hemorrhagic nature of the lesions is consistent with the fact that many children infected with EAggEC experience bloody diarrhea (Bhan et al., 1989, and Vial et al., 1988). In the study of Haque et al., 1994, it seems that bacteria, because of the property of aggregative adherence, may serve to create a microenvironment whereby contact hemolysins are secreted in close contact to the erythrocytes. In addition to heat-stable and heat-labile toxin produced by EAggEC strains, plasmid-mediated and thermoregulated contact hemolysin production may be a factor for the pathogenesis of these organisms (Haque et al., 1994).

4. The genetics and properties of β-glucuronidase (GUS) in *E. coli.*

Since there is the report that enzyme β-Glucuronidase (GUS) activity was mostly limited to *E. coli* (94-97%) (Kilian et al., 1976, 1979), many methods used in assaying for *E. coli* from various sources have been developed by based wholly or in part on GUS detection (Frampton et al., 1993). GUS is the first enzyme of the hexuronide-hexuronate pathway in *E. coli* and the pathway was shown in Fig. 1 (Novel et al., 1976a). GUS activity of *E. coli* plays a specific and very important roles in the gut which is the natural habitat of *E. coli*. In the liver of vertebrates, one

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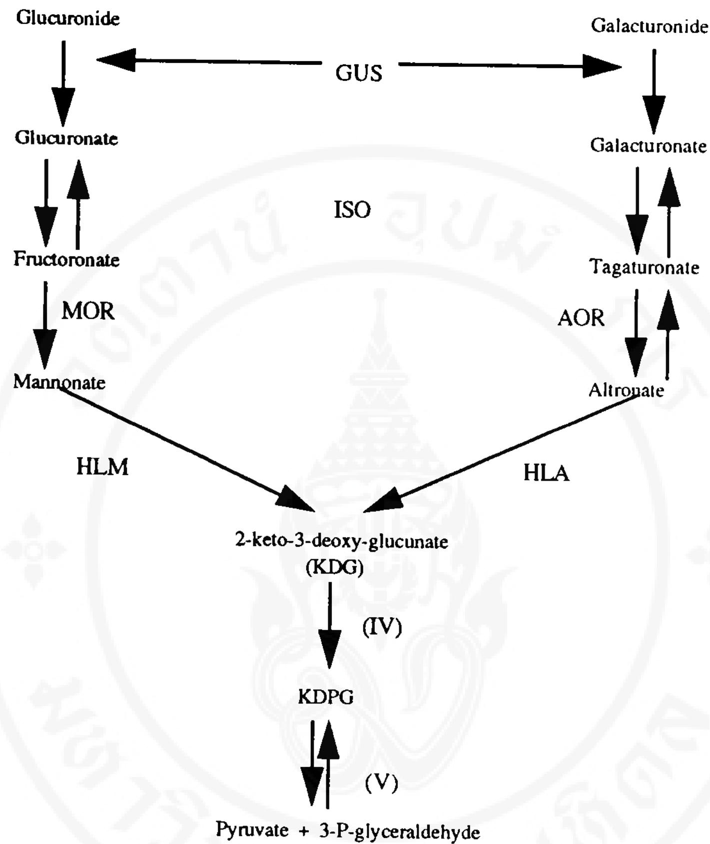

GUS = β-Gluguronidase, ISO = Uronate Isomerase, MOR = Mannonate oxidoreductase,
AOR = Altronate oxidoreductase, HLA = Altronate Hydrolyase, HLM = Mannonate Hydrolyase,
IV = 2-keto-3 -deoxy gluconate (KDG) kinase, V = 2-keto-3-deoxygluconate-6 phosphate (KDPG) aldolase

Fig. 1 The hexuronide - hexuronate pathway in *E. coli.* Glucuronide or galacturonide is converted to 2-keto-3-deoxyglucunate by passing through different enzyme reaction steps. A 2-keto-3-deoxy-glucunate is then processed to be pyruvate and 3-P-glyceraldehyde.

(From Novel et al., 1976a)

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of the major pathways of detoxification of endogenous and xenobiotic organic compounds so called aglycone, is carried out by conjugation to glucuronic acid. The active form of aglycone then become aglycone-β-D-glucuronide, inactive form, which is more water soluble and able to be excreated in the bile or the urine (see Fig. 2) (Wilson et al., 1992). Thus, the gut is a rich source of glucuronic acid compounds, providing as a carbon source that can be efficiently utilized by *E. coli*. Glucuronide substrates are taken up by *E. coli* via a specific transporter, the glucuronide permease, cleaved by β-glucuronidase, and the glucuronic acid residue thus released is used as a carbon source. In general, the aglycone component of the glucuronide substrate is not used by *E. coli* and passes back across the bacterial membrane into the gut and is reabsorbed into the bloodstream. This circulation in the liver and deglucuronidation in the gut is termed enterohepatic circulation (Fig. 2) (Wilson et al., 1992). This phenomenon is of great physiological important because it means that, due in large part to the action of microbial β-glucuronidase, many compounds, including endogenous steroid hormones and exogenously administered drugs, are not eliminated from the body all at once. Rather, the levels of these compounds in the bloodstream oscillate due to this circulatory process (Wilson et al., 1992). This process is of great significant in determining pharmaceutical dosages, and indeed some drugs are specifically administered as the glucuronide conjugate, relying on the action of β-glucuronidase to release the active aglycone. Enterohepatic circulation is also important in the day-to-day physiological state of the body, probably being a prime cause of the physiological impact of variations in diet or in gut flora (Wilson et al., 1992)

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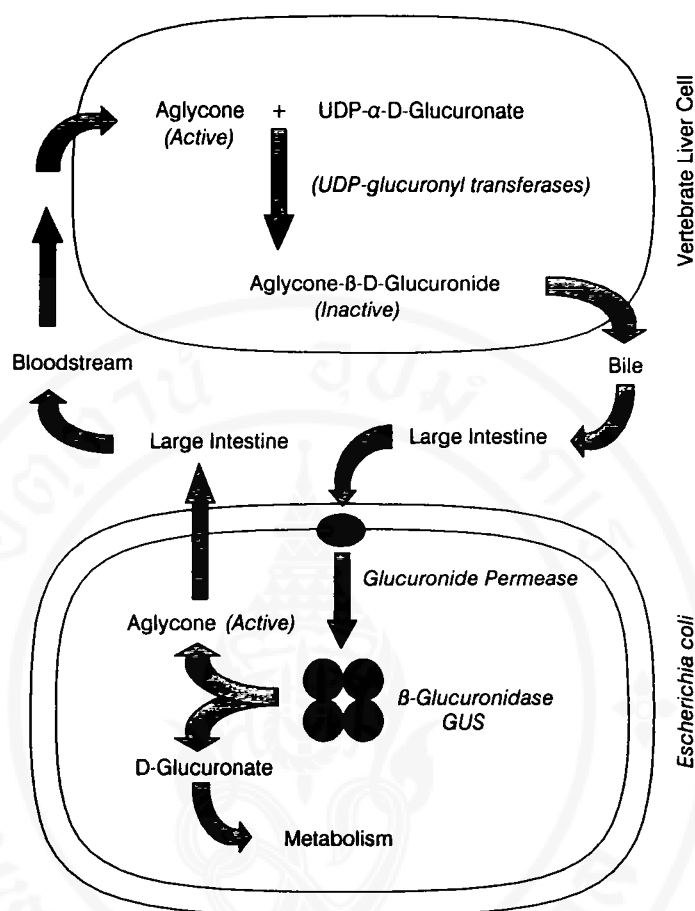

Fig. 2 Schematic representation of enterohepatic circulation. The endogenous and xenobiotic organic compounds or so called aglycon is detoxified by conjugating with glucuronic acid. The conjugative form (aglycone-β-D-glucuronide) is more water soluble. See text for more detail description.

(From Wilson et al., 1992)

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GUS activity is also found in certain other bacterial species such as nonenterobacterial, anaerobic residents of the gut, primarily in *Bacteroides* and *Clostridium* species (Hawkesworth et al., 1971). These species exhibit lower β-glucuronidase activity per cell than *E. coli*. It is not know whether these organisms possess a glucuronide permease and whether their GUS activity, or any permease activity, possesses the same substrate versatility as those of *E. coli*. *Shigella* species, an *E. coli* very closely related bacteria , are also found to produce GUS (Wilson et al., 1992).

The gene encoding β-glucuronidase, *uidA*, maps at minute 36 on the *E. coli* chromosome, between the loci *add* (adenine deaminase) and *manA* (mannose-6-phosphate isomerase) genes (Jochimsen et al., 1975). It has become clear that other genes involved in glucuronide metabolism and in regulation of β-glucuronidase activity map to the same region of the *E. coli* chromosome, forming the *uid* operon. The model of the structure and functioning of the *uid* operon is summarized in Fig. 3 (Wilson et al., 1992). There are two genes downstream of *uidA*, one of which, *uidB*, encodes a glucuronide-specific permease, and the third gene, *uidC*, encodes the product with unknown function. Upstream of *uidA*, and separately transcribed, is a gene, *uidR*, encoding a specific repressor of the *uid* operon (Wilson et al., 1992). DNA sequence of *uidA* gene studied by Jefferson et al., 1986 was shown in Fig. 4.

Genetic evidence suggests that β-glucuronidase activity is not constitutively expressed in *E. coli* and there are three different factors regulating transcription of the operon. The primary mechanism of control is induction by glucuronide substrates.

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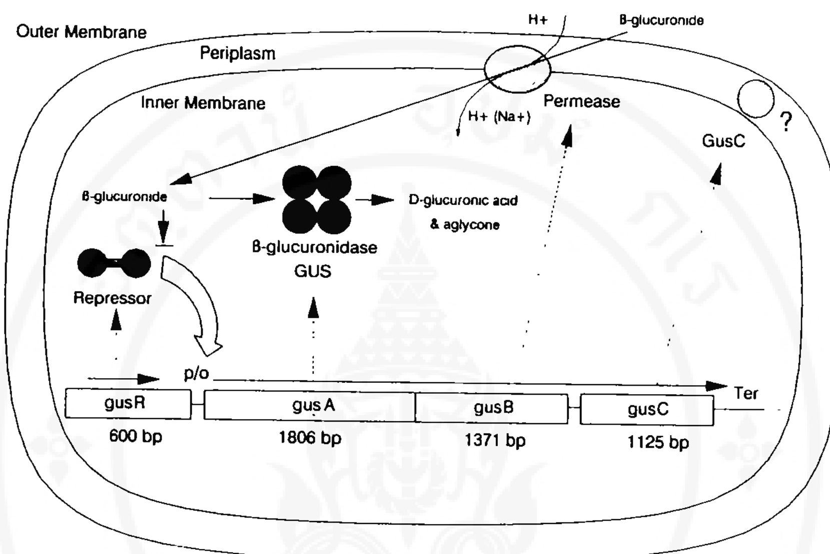

Fig. 3 The *uid* operon in *E. coli*. The operon compose of 4 genes, *uidR* (*gusR*), *uidA* (*gusA*), *uidB* (*gusB*), and *uidC* (*gusC*). The *uidA*, *B*, and *C* genes are proposed to be transcribed as a single mRNA.

(From Wilson et al., 1992)

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                                                                                              100
GAATTCCCCCCAAAATATTCACTGTAGCCATATGTCATGAGAGTTTATCGTTCCCAATACGCTCGAACGAACGTTCGGTTGCTTATTTTATGGCTTCTGTCAACGCTGTTTTAAAGATTA
                                                                                    200
ATGCGATCTATATCACGCTGTGGGTATTGCAGTTTTTGGTTTTTTGATCGCGGTGTCAGTTCTTTTTATTTCCATTTCTCTTCCATGGGTTTCTCACAGATAACTGTGTGCAACACAGA
                                                      300
ATTGGTTAACTAATCAGATTAAAGGTTGACCAGTATTATTATCTTAATGAGGAGTCCCTTATGTTACGTCCTGTAGAAACCCCAACCCGTGAAATCAAAAAACTCGACGGCCTGTGGGCA
                                                              MetLeuArgProValGluThrProThrArgGluIleLysLysLeuAspGlyLeuTrpAla
                                  400
TTCAGTCTGGATCGCGAAAACTGTGGAATTGATCAGCGTTGGTGGGAAAGCGCGTTACAAGAAAGCCGGGCAATTGCTGTGCCAGGCAGTTTTAACGATCAGTTCGCCGATGCAGATATT
PheSerLeuAspArgGluAsnCysGlyIleAspGlnArgTrpTrpGluSerAlaLeuGlnGluSerArgAlaIleAlaValProGlySerPheAsnAspGlnPheAlaAspAlaAspIle
                  500
CGTAATTATGCGGGCAACGTCTGGTATCAGCGCGAAGTCTTTATACCGAAAGGTTGGGCAGGCCAGCGTATCGTGCTGCGTTTCGATGCGGTCACTCATTACGGCAAAGTGTGGGTCAAT
ArgAsnTyrAlaGlyAsnValTrpTyrGlnArgGluValPheIleProLysGlyTrpAlaGlyGlnArgIleValLeuArgPheAspAlaValThrHisTyrGlyLysValTrpValAsn
600                                                                                                   700
AATCAGGAAGTGATGGAGCATCAGGGCGGCTATACGCCATTTGAAGCCGATGTCACGCCGTATGTTATTGCCGGGAAAAGTGTACGTATCACCGTTTGTGTGAACAACGAACTGAACTGG
AsnGlnGluValMetGluHisGlnGlyGlyTyrThrProPheGluAlaAspValThrProTyrValIleAlaGlyLysSerValArgIleThrValCysValAsnAsnGluLeuAsnTrp
                                                                                     800
CAGACTATCCCGCCGGGAATGGTGATTACCGACGAAAACGGCAAGAAAAAGCAGTCTTACTTCCATGATTTCTTTAACTATGCCGGGATCCATCGCAGCGTAATGCTCTACACCACGCCG
GlnThrIleProProGlyMetValIleThrAspGluAsnGlyLysLysLysGlnSerTyrPheHisAspPhePheAsnTyrAlaGlyIleHisArgSerValMetLeuTyrThrThrPro
                                                              900
AACACCTGGGTGGACGATATCACCGTGGTGACGCATGTCGCGCAAGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGTCAGCGTTGAACTGCGTGATGCGGAT
AsnThrTrpValAspAspIleThrValValThrHisValAlaGlnAspCysAsnHisAlaSerValAspTrpGlnValValAlaAsnGlyAspValSerValGluLeuArgAspAlaAsp
                                            1000
CAACAGGTGGTTGCAACTGGACAAGGCACTAGCGGGACTTTGCAAGTGGTGAATCCGCACCTCTGGCAACCGGGTGAAGGTTATCTCTATGAACTGTGCGTCACAGCCAAAAGCCAGACA
GlnGlnValValAlaThrGlyGlnGlyThrSerGlyThrLeuGlnValValAsnProHisLeuTrpGlnProGlyGluGlyTyrLeuTyrGluLeuCysValThrAlaLysSerGlnThr
                        1100
GAGTGTGATATCTACCCGCTTCGCGTCGGCATCCGGTCAGTGGCAGTGAAGGGCGAACAGTTCCTGATTAACCACAAACCGTTCTACTTTACTGGCTTTGGTCGTCATGAAGATGCGGAC
GluCysAspIleTyrProLeuArgValGlyIleArgSerValAlaValLysGlyGluGlnPheLeuIleAsnHisLysProPheTyrPheThrGlyPheGlyArgHisGluAspAlaAsp
1200                                                                                                  1300
TTACGTGGCAAAGGATTCGATAACGTGCTGATGGTGCACGACCACGCATTAATGGACTGGATTGGGGCCAACTCCTACCGTACCTCGCATTACCCTTACGCTGAAGAGATGCTCGACTGG
LeuArgGlyLysGlyPheAspAsnValLeuMetValHisAspHisAlaLeuMetAspTrpIleGlyAlaAsnSerTyrArgThrSerHisTyrProTyrAlaGluGluMetLeuAspTrp
                                                                                     1400
GCAGATGAACATGGCATCGTGGTGATTGATGAAACTGCTGCTGTCGGCTTTAACCTCTCTTTAGGCATTGGTTTCGAAGCGGGCAACAAGCCGAAAGAACTGTACAGCGAAGAGGCAGTC
AlaAspGluHisGlyIleValValIleAspGluThrAlaAlaValGlyPheAsnLeuSerLeuGlyIleGlyPheGluAlaGlyAsnLysProLysGluLeuTyrSerGluGluAlaVal
                                                              1500
AACGGGGAAACTCAGCAAGCGCACTTACAGGCGATTAAAGAGCTGATAGCGCGTGACAAAAACCACCCAAGCGTGGTGATGTGGAGTATTGCCAACGAACCGGATACCCGTCCGCAAGTG
AsnGlyGluThrGlnGlnAlaHisLeuGlnAlaIleLysGluLeuIleAlaArgAspLysAsnHisProSerValValMetTrpSerIleAlaAsnGluProAspThrArgProGlnVal
                                            1600
CACGGGAATATTTCGCCACTGGCGGAAGCAACGCGTAAACTCGACCCGACGCGTCCGATCACCTGCGTCAATGTAATGTTCTGCGACGCTCACACCGATACCATCAGCGATCTCTTTGAT
HisGlyAsnIleSerProLeuAlaGluAlaThrArgLysLeuAspProThrArgProIleThrCysValAsnValMetPheCysAspAlaHisThrAspThrIleSerAspLeuPheAsp
                        1700
GTGCTGTGCCTGAACCGTTATTACGGATGGTATGTCCAAAGCGGCGATTTGGAAACGGCAGAGAAGGTACTGGAAAAAGAACTTCTGGCCTGGCAGGAGAAACTGCATCAGCCGATTATC
ValLeuCysLeuAsnArgTyrTyrGlyTrpTyrValGlnSerGlyAspLeuGluThrAlaGluLysValLeuGluLysGluLeuLeuAlaTrpGlnGluLysLeuHisGlnProIleIle
1800                                                                                                  1900
ATCACCGAATACGGCGTGGATACGTTAGCCGGGCTGCACTCAATGTACACCGACATGTGGAGTGAAGAGTATCAGTGTGCATGGCTGGATATGTATCACCGCGTCTTTGATCGCGTCAGC
IleThrGluTyrGlyValAspThrLeuAlaGlyLeuHisSerMetTyrThrAspMetTrpSerGluGluTyrGlnCysAlaTrpLeuAspMetTyrHisArgValPheAspArgValSer
                                                                                     2000
GCCGTCGTCGGTGAACAGGTATGGAATTTCGCCGATTTTGCGACCTCGCAAGGCATATTGCGCGTTGGCGGTAACAAGAAAGGGATCTTCACTCGCGACCGCAAACCGAAGTCGGCGGCT
AlaValValGlyGluGlnValTrpAsnPheAlaAspPheAlaThrSerGlnGlyIleLeuArgValGlyGlyAsnLysLysGlyIlePheThrArgAspArgLysProLysSerAlaAla
                                                              2100 ORF-------->
TTTCTGCTGCAAAAACGCTGGACTGGCATGAACTTCGGTGAAAAACCGCAGCAGGGAGGCAAACAATGAATCAACAACTCTCCTGGCGCACCATCGTCGGCTACAGCCTCGGTGACGTCG
PheLeuLeuGlnLysArgTrpThrGlyMetAsnPheGlyGluLysProGlnGlnGlyGlyLysGln***
                                            2200
CCAATAACTTCGCCTTCGCAATGGGGGCGCTCTTCCTGTTGAGTTACTACACCGACGTCGCTGGCGTCGGTGCCGCTGGCGGGGCACATGCTGTTACTGGTGCGGGTATTCGATGCCTTC
                        2300
GCCGACGTCTTTGCCGGACGAGTGGTGGACAGTGTGAATACCGCTGGGGAAAATTCCGCCCGTTTTTACTCTTCGGTACTGGCGCGTTAATGATCTTCAGCGTGCTGGTATTCTGGGTGC
2400                                      2439
TGACCGACTGGAGCCATGGTAGCAAAGTGGTGTATGCATG
```

Fig. 4 DNA sequence of the 2439 bp fragment containing the *uidA* gene. The arrows before the coding sequence indicate regions of dyad symmetry that could be recognition sequences for effector molecules. The overlined region is the putative Shine-Dalgano (ribosome binding site) sequence for the *uidA* gene; brackets indicate two possible Pribnow boxes. All of the palindromic regions fall within the smallest subcloned region (from the *Sau*3A site at 166 to *Hinf*I site at 291) that gave constitutive genomic expression of *uidA* when present in high copy *In trans*, consistent with their proposed function as repressor binding sites. The terminator codon at 2106 overlaps with an ATG that may be the initiator codon of a second open reading frame.

(From Jefferson et al., 1986)

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This regulation is due to the action of the product of the *uidR* gene, which encodes a repressor that is specific for the *uid* operon. Inactivation or deletion of *uidR* leads to constitutive β-glucuronidase activity (Novel et al., 1976a). The *uidR* repressor acts by binding to *uidA* operator sequences so prevention of transcription, this repression being relieved when a glucuronide substrate binds to the repressor and inactivates it. A second key level of control is that of cyclic AMP-dependent catabolite repression. A putative CAP binding site has been identified in the *uidA* upstream sequences (Jefferson et al., 1986). A third level of regulation is a weak negative control on *uidA* expression by *uxuR* gene, which maps elsewhere on the *E. coli* chromosome at minute 98 (Novel et al., 1976b).

There are reports of GUS activity in strains of *Streptococcus*, *Staphylococcus, Corynebacteria* and certain bacteria associated with plants such as the bacteria closely resembles the *Myxobacteria*, *Micrococcus* or *Clavibacter*, *Brevibacterium* and *Curtobacterium* species (Wilson et al., 1992). In addition, there are reports of GUS activity in some *Salmonella* species (Kilian et al., 1979) but these appear not to have been confirmed by later studies (e.g., Perez et al., 1986, and Cleuziat et al., 1990). Since *E. coli* and *Shigella* are serologically related and do exchange genetic information via intergenetic conjugation, at least among the *Enterobacteriaceae*, GUS activity can reasonably be said to be restricted to a single taxonomic group that of *E. coli* and *Shigella* species (Wilson et al., 1992).

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5. Standard allowance of the amount of bacteria in drinking water and food.

5.1 Standard allowance of the amount of bacteria in drinking water.

The concern about transmission of disease through water has triggered the awareness of requirement for water purity. Therefore, tests have been developed to determine the safety of water ; many of these tests are also applicable to foods. It is not practical, however, to look only for pathogens in water supplies. For one thing, if the pathogen causing typhoid or cholera were subjected for detection in the water system, the discovery would already be too late to prevent an outbreak of the disease. Moreover, such pathogens would probably be present only in small numbers and might not be included in tested samples and negative results will be obtained. The tests for water safety in use today are aimed instead at detecting particular indicator organisms (Tortora et al., 1989). There are several criteria for an indicator organism. The following are among the suggested criteria for such an indicator (Prescott et al., 1993):

1.) The indicator bacterium should be suitable for the analysis of all types of water : tap, river, ground, impounded, recreational, estuary, sea, and waste.

2.) The indicator bacterium should be present whenever enteric pathogens are present.

3.) The indicator bacterium should survive longer than the hardiest enteric pathogen.

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4.) The indicator bacterium should not reproduce in the contaminated water, thereby leading to an inflated value.

5.) The assay procedure for the indicator should have great specificity; in other words, other bacteria should not give positive results. In addition, the procedure should have high sensitivity and detect low levels of the indicator.

6.) The testing method should be easy to perform.

7.) The indicator should be harmless to humans.

8.) The level of the indicator bacterium in contaminated water should have some direct relationship to the degree of fecal pollution.

The most important criterion is that the organism be consistently present in human feces in substantial numbers so that its detection will be a good indication that human wastes are entering the water. In the United States, the usual indicator organisms are the coliform bacteria, including *E. coli*, *Enterobacter aerogenes*, and *Klebsiella pneumoniae* (Prescott et al., 1993). Coliforms are members of the family *Enterobacteriaceae* and defined as aerobic or facultatively anaerobic, gram-negative, nonendospore-forming, rod-shaped bacteria that ferment lactose to form gas within 48 hours of being placed in lactose broth at 35°C. Because some coliforms are not solely enteric bacteria but are more commonly found in plant and soil samples, therefore, many standards for food and water specify the determination of fecal coliforms. The predominant fecal coliform is *E. coli*, which constitutes a large proportion of the human intestinal population (Prescott et al., 1993). It is important to note that coliforms are not themselves pathogenic under normal condition, although they can cause diarrhea and opportunistic urinary tract infection. When "foreign" enteric indicator bacteria, such *E. coli*, is not detectable according to the regulations, a water is considered drinkable, or suitable for human consumption. US federal regulation, for

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example, require that fecal coliforms must be not detected in 100 ml of drinking water (Prescott et al., 1993 and Frazier et al., 1988). In Thailand, the regulation is also the same as in USA that *E. coli* must not be detected in 100 ml of drinking water and coliforms must be detected less than 2.2 in 100 ml of drinking by using Most Probable Number method (Data from Ministry of Public Health, 1981).

5.2 Standard allowance of the amount of bacteria in food.

The objectives of the control, regulation, and inspection of food are primarily to give assurance that foods received by the consumer will be pure, healthful and of the quality claimed. Food regulation and control is the concern of all countries, and it is therefore inevitable that many of the standards or regulations developed are quite different from one country to another (Frazier, 1988). Standards usually are based on total numbers of organisms, numbers of an indicator organism, or numbers (or total absence) of pathogens and must be adapted to the types of food for which they are intended. The type of pathogen most likely to be present will be different in different food. Tests for coliform bacteria, for example, to indicate the possible presence of intestinal pathogens are useful in setting standards for oysters but have little meaning for frozen orange juice (Frazier, 1988). Acceptance or rejection of a lot will be based on some microbiological test performed from several sample units (ICMSF, 1989). The letters symbols, n, c, m, and M, represented various numbers are used to define the decision-making process. The letter "n" is the number of sample units analyzed which are chosen separately and independently. The maximum allowable number of sample units yielding unsatisfactory test results, e.g., the presence of the organism, is

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represented by the letter "c". The symbol "m" is used to represent a microbiological criterion that in a 2-class plan separates good quality (value equal to or less than m) from defective quality (value above m), or in a 3-class plan separates good quality from marginally acceptable quality while "M" is a microbiological criterion that in a 3-class plan separates marginally acceptable quality from defective quality. Value at or above M are unacceptable (ICMSF, 1989, and Frazier et al., 1988). Counts between m and M are undesirable, but some such counts can be accepted if there are not too many of them. For example, number of faecal coliform per gram in fresh or frozen fish products are n=10, c=3, m=1,000 and M=10,000 which elaborate as ; take a sample of 10 sample units and make a test on each unit ; then if 3 samples or fewer show the presence of the organism that do not exceed M, accept the lot, but if 4 or more of the 10 show the presence of the organism, reject the lot. The four numbers of n, c, m, and M may vary from the types of food and the quality approval by different countries. For example, the microbiological criteria for presence of *E. coli* applicable to the production of cooked shrimp to European communities determines that m=10, M=100 n=5, and c=1 which means that if 1 sample show the present of *E. coli* that do not exceed 100, accept the lot, but if more than 1 show the presence of *E. coli* exceed 100, reject the lot. Fish and fishery products exported to New Zealand, specification for fecal coliform per gram of all sample units is determined that n=5, c=2, m=20, and M=200 while the value determined to Norway is n=5, c=3, m=3, and M=10 (Data obtained from Food for Export Promotion Section. Division of Food for Export Analysis, 1993). The other examples were microbiological regulation of frozen shrimp which determined by foreign buyer or foreign government shown in Table 2.

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Table 2 Microbiological regulation of frozen shrimp product.

Type of frozen product	Country	Regulation of microbiological standard quality
Shrimp	Australia	Total plate count/g n=5, c=2, m=10^5, M=10^6
		E. coli/g n=5, c=2, m=9, M=70
		Salmonella/25 g n=5, c=0, m=0
		Staphylococcus aureus/g n=5, c=1, m=nd in 0.01g, M=nd in 0.001 g
	Belgium	Total plate count/g=10^5
		Coliform/g=10
		Salmonella/25g nil
		Shigella/25 g nil
		Staphylococcus aureus/g=100
	France	Total plate count/g=10^5
		E. coli /g=10
		Salmonella/25g nil
		Staphylococcus aureus/g=100
	Malaysia	Total plate count/g =10^5
		E. coli/g =70
		Salmonella/25g nil
		Staphylococcus aureus/g not detected in 0.01
	New Zealand	Total plate count/g n=5, c=2, m=10^5, M=10^6
		E. coli/g n=5, c=2, m=20, M=200
		Salmonella/25g n=5, c=0, m=0
		Staphylococcus aureus/g n=5, c=2, m=100, M=10^6
	Spain	Total plate count/g=10^6
		E. coli/g=250
		Salmonella/25 g nil
	U.K.	*E. coli*/g less than 10 per gram (0.1 ml tubes negative) but allowing one failure, out of the samples taken, within the range 10-100 (0.1 tubes positive but 0.01 ml tubes negative)
		Staphylococcus aureus less than 1,000 but allowing one failure, out of the samples taken, with the range 1,000-10,000.
	U.S.A.	TPC/g=5x10^5 (raw), 2x10^5 (cooked), Coliform/g=100
		E. coli/g negative, *Salmonella*/25 g absent
		Vibrio parahaemolyticus/g absent

(Data obtained from Food for Export Promotion Section. Division of Food for Export Analysis, 1993)

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6. Methods for detection of *E. coli* in drinking water or in foods.

To test for the present of *E. coli*, a variety of simpler and more specific tests have been developed.

6.1 Multiple tube fermentation method.

This is a technique for determining the most probable number (MPN) of bacteria in water and was first described by McCrady in 1915. This method consists of the three steps, presumptive test, confirmed test and completed test. Presumptive test is inoculating known volumes of a water sample (use five 10-ml sample portions or five 100-ml sample portions) (American Public Health Associate, 1976, American Public Health Associate, 1990) or food sample (25 g of food mixed with 225 ml of broth and prepared dilutions in range 1:10, 1:100, and 1:1000, and higher if necessary) (AOAC, 1990) into tubes of selective lactose broth consisted of beef extract, peptone, and lactose. The tubes are examined for growth and production of acid and gas after 24 to 48 hours of incubation at 35°C. After interpretation , positive tubes are subjected to confirmed test by being inoculated into Brilliant green lactose bile broth (BGB) consisted of peptone, lactose, oxgall, and brilliant green and incubated for 48 hours at 35^0C to confirm the present of coliform group or being inoculated into EC broth consisted of tryptose, lactose, bile salt mixture, dipotassium hydrogen phosphate, potassium dihydrogenphosphate, sodium chloride and incubated for 24-48 hours at 44°C to confirm the present of *E. coli*. Confirmation of *E. coli* is completed by streaking samples from gas-positive EC broth tubes onto Levine eosin-methylene blue

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(EMB) agar and typical colonies are tested by a series of biochemical tests including indole, methyl red, Voges-Proskaur, citrate (IMVIC). The results were computed as MPN of *E. coli* per g (or per ml if liquid product) in addition to considering the bacteria as gram negative, nonspore forming rods, producing gas in lactose and producing ++-- or -+-- IMVIC patterns (American Public Health Associate, 1976, American Public Health Associate, 1990). Table 3 and Table 4 showed the examples of MPN tables when five 10-ml portions (water) and 3 tubes with each of 0.1, 0.01, and 0.001 g portions (food) are used, respectively. In food and dairy product analysis, the MPN assay is also accepted (Feng et al., 1982). Final identification may take as long as 4-6 days. The disadvantage of these procedures are laborious, and time consuming. Debilitated *E. coli* cells are sensitive to high incubation temperatures e.g. 44°C and to selective media used for their detection and enumeration. In addition, the MPN assay is susceptible to bacterial interference ; false negative reactions (absence of gas production in the present of coliforms) may occur in the presumptive, confirmed, and completed stages of the MPN analysis (Feng et al., 1982). Other factors such as synergistic gas production from lactose by non coliforms, cultivation of anaerogenic and non-lactose-fermenting *E. coli* strains, and the presence of lactose-fermenting noncoliforms, have contributed to this inefficiency of *E. coli* detection method (Feng et al., 1982)

6.2 Membrane filter method.

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Membrane filtration is used for the determination of microbe numbers in gases , fluids and solid substances. In the latter case, a homogeneous suspension must be prepared (Kiss I, 1984). In this technique, a known volume of water or food sample

Table 3 MPN Index and 95% confidence limits for various combinations of positive and negative results when five 10-ml portions are used.

No. of Tubes Giving Positive Reaction out of 5 of 10 ml Each	MPN Index/100 ml	95% Confidence Limits (Approximate)	
		Lower	Upper
0	< 2.2	0	6.0
1	2.2	0.1	12.6
2	5.1	0.5	19.2
3	9.2	1.6	29.4
4	16.0	3.3	52.9
5	>16.0	8.0	Infinite

(From American Public Health Associate, 1976, American Public Health Associate, 1990).

Table 4 Most Probable Numbers (MPN) per 1g of Sample, Using 3 Tubes with Each of 0.1, 0.01, and 0.001 g Portion.

Positivo Tubes				Positive Tubes				Positivo Tubes				Positivo Tubes			
0 1	0 01	0 001	MPN	0.1	0 01	0 001	MPN	0 1	0 01	0 001	MPN	0 1	0 01	0 001	MPN
0	0	0	<3	1	0	0	3 6	2	0	0	9 1	3	0	0	23
0	0	1	3	1	0	1	7 2	2	0	1	14	3	0	1	39
0	0	2	6	1	0	2	11	2	0	2	20	3	0	2	64
0	0	3	9	1	0	3	15	2	0	3	26	3	0	3	95
0	1	0	3	1	1	0	7 3	2	1	0	15	3	1	0	43
0	1	1	6 1	1	1	1	11	2	1	1	20	3	1	1	75
0	1	2	9 2	1	1	2	15	2	1	2	27	3	1	2	120
0	1	3	12	1	1	3	19	2	1	3	34	3	1	3	160
0	2	0	6 2	1	2	0	11	2	2	0	21	3	2	0	93
0	2	1	9 3	1	2	1	15	2	2	1	28	3	2	1	150
0	2	2	12	1	2	2	20	2	2	2	35	3	2	2	210
0	2	3	16	1	2	3	24	2	2	3	42	3	2	3	290
0	3	0	9 4	1	3	0	16	2	3	0	29	3	3	0	240
0	3	1	13	1	3	1	20	2	3	1	36	3	3	1	460
0	3	2	16	1	3	2	24	2	3	2	44	3	3	2	1100
0	3	3	19	1	3	3	29	2	3	3	53	3	3	3	>1100

(AOAC, 1990)

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is passed through a membrane filter (American Public Health Associate, 1990, and Sharpe et al., 1979). The filter with its trapped bacteria is transferred to the surface of a solid medium or to an absorptive pad containing the desired liquid medium. Use of the proper medium allows the rapid detection of total coliforms or *E. coli* by the presence of their characteristic colonies. For example, all organisms that produce red colonies with a metallic sheen on M-Endo or LES-Endo agar within 24 hours of incubation are considered members of the coliform group (Singh et al., 1992) or fluorescence and indole positive colonies on Fluorocult media (Merk) are considered as *E. coli*. Verification of colonies observed after membrane filtration by biochemical tests is advisable. The disadvantages of this method are that high-turbidity water or food limit volumes sample, high populations of background bacteria cause overgrowth, metals and phenols can adsorb to filters and inhibit growth (Prescott et al., 1993).

6.3 Presence-Absence (P-A) test.

More simplified tests for detecting coliforms and fecal coliforms are available. The presence-absence test (P-A test) was used for detection of coliforms in water. This is a modification of the MPN procedure, in one P-A technique, 100 ml water sample are incubated at 37°C in a single culture bottle with a triple-strength P-A broth containing lactose broth, lauryl tryptose broth and bromocresol purple indicator. The P-A test is based on the assumption that no coliforms should be present in 100 ml of drinking water. The samples are examined after 24 and 48 hours for the production of acid and gas. A positive test results in the production of acid (a yellow color) and constitutes a positive presumptive test still requiring confirmation. Detection of fecal

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coliforms, fecal steptococci, and other indicator bacteria by the P-A test may be achieved by appropriate selection of confirmatory media and incubation time and temperature. For example, the presence of coliforms was confirmed by transfering cultures that show acid reaction or acid and gas reaction to brilliant green lactose bile (BGLB) broth for incubation at 35°C while the confirmation of the present of fecal coliforms or *E. coli* was processed by using EC broth and incubation at 44°C (American Public Health Associate, 1990).

6.4 Detection of *E. coli* by methods based on detection of the enzyme GUS.

Since the enzyme GUS is specifically present in *E. coli* strains. About 94-97% of *E. coli* is GUS-positive. The other bacteria except *Shigella* are generally GUS-negative (Kilian et al., 1976, 1979). Therefore, in the last decade of the 20th century, cultural tests based wholly or in part on GUS detection have been developed and used in assaying for *E. coli*. 4-Methylumbelliferyl β-D-glucuronic acid (MUG) is the most widely used fluorogenic substrate for detection of GUS activity in vitro (Fig. 5). GUS hydrolyzes β-linked-D-glucuronide to D-glucuronic acid and 4-methylumbelliferone (MU) that can be detected by long wavelength (366 nm) UV light (Wilson et al., 1992). The incorporation of MUG in standard media as a sensitive means of detecting *E. coli* in foods and water was introduced by Feng et al., 1982. The use of enzyme activity profiles as a supplement to the standard methods has led to the development of more rapid assay systems for *E. coli* in foods and water. For example, when MUG was incorporated in lauryl sulfate tryptose broth (LST-MUG), the presumptive medium in an MPN assay, 91% of the tubes showing both gas

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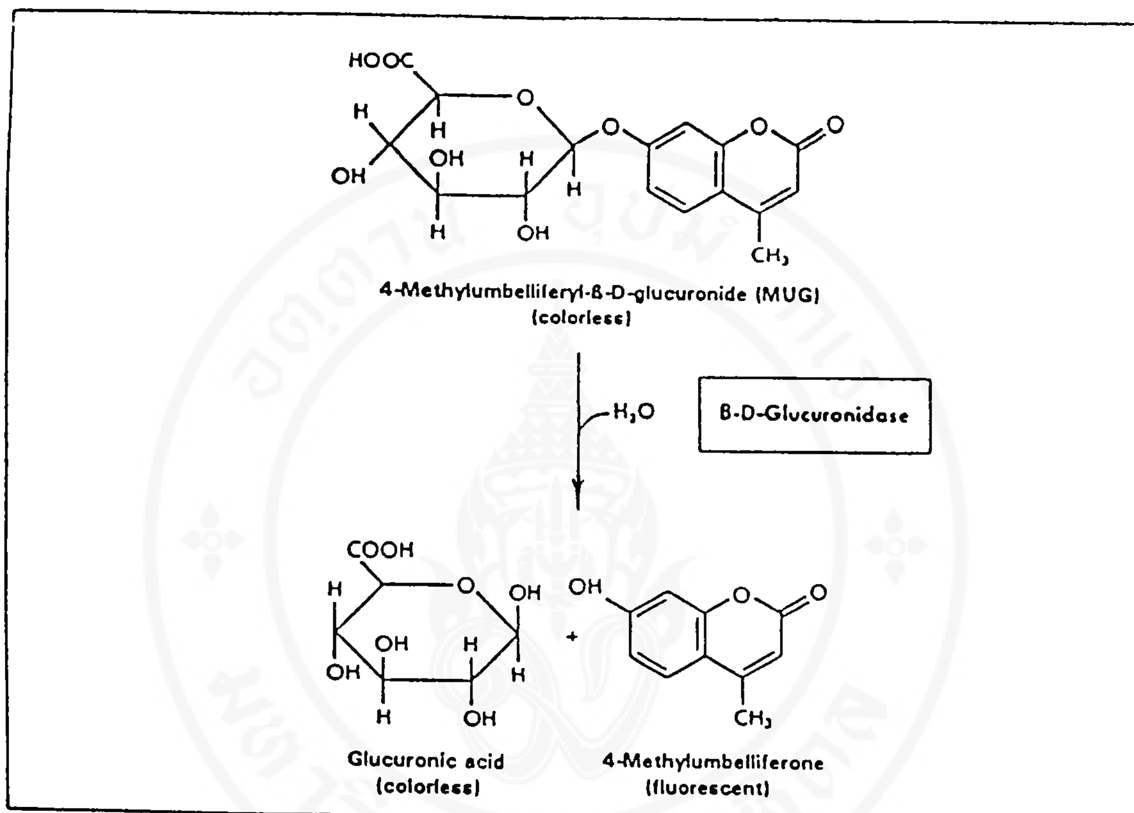

Fig. 5 Reaction of β-D-glucuronidase (GUS) with 4-methylumbelliferyl β-D-glucuronic acid (MUG). The enzyme will cleave MUG and liberates 4-methylumbelliferone (MU) which is fluorescent.

(From Wilson et al., 1992)

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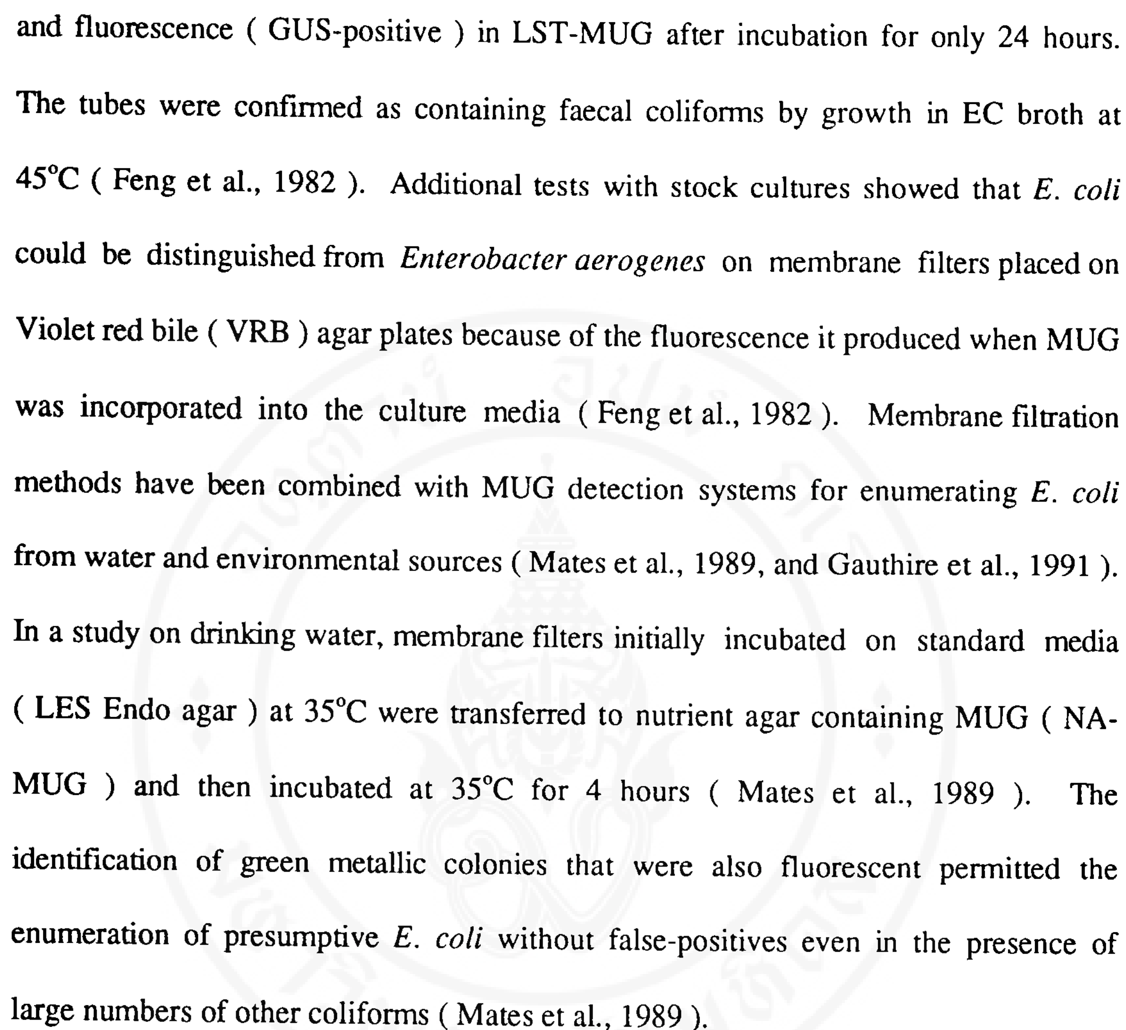

and fluorescence (GUS-positive) in LST-MUG after incubation for only 24 hours. The tubes were confirmed as containing faecal coliforms by growth in EC broth at 45°C (Feng et al., 1982). Additional tests with stock cultures showed that *E. coli* could be distinguished from *Enterobacter aerogenes* on membrane filters placed on Violet red bile (VRB) agar plates because of the fluorescence it produced when MUG was incorporated into the culture media (Feng et al., 1982). Membrane filtration methods have been combined with MUG detection systems for enumerating *E. coli* from water and environmental sources (Mates et al., 1989, and Gauthire et al., 1991). In a study on drinking water, membrane filters initially incubated on standard media (LES Endo agar) at 35°C were transferred to nutrient agar containing MUG (NA-MUG) and then incubated at 35°C for 4 hours (Mates et al., 1989). The identification of green metallic colonies that were also fluorescent permitted the enumeration of presumptive *E. coli* without false-positives even in the presence of large numbers of other coliforms (Mates et al., 1989).

In addition to the works of individual investigators who combined GUS substrates with various selective and differential media, commercial test kits have been developed that are capable of rapidly measuring GUS activity alone or, in conjunction with other enzymes, providing multi-enzyme profiles (Frampton et al., 1993). Some of the commercial tests currently used are described as follow. Rosco tablets (Rosco Diagnostica , Taastrup , Denmark) containing para-nitrophenyl-β-D-glucuronide (PNPG) were used to evaluate for identify *E. coli* in both clinical and environmental isolates. Fluorocult media containing MUG (E. Merk AG, Darmstadt, Germany) also evaluated *E. coli* in water, food, and clinical isolates. The Rapid Identification Method

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(RIM) *Escherichia coli* Kit (Austin Biologicals Laboratories Inc., Austin, TX) evaluated this bacteria on clinical isolates (Varga et al., 1986 and Edberg et al., 1986). The kit includes a tube of buffer plus reagent impregnated swab containing 0-nitrophenyl-β-D-galactoside (ONPG) and MUG for detecting β-galactosidase and GUS, respectively, with tryptophanase (indole) detected by Kovac's reagent. The Rapid Detect *E. coli* (Organon Teknika, West Orange, NJ) was similar to the RIM *E. coli* Kit but provides the same substrates on paper disks instead of swabs. In these tests *E. coli* gives positive results with all the above stated three enzymes. Two other ONPG-MUG prepared media products which are Colilert (Access Analytical, Branford, CN) and Coliquick (Hach Co., Loveland, CO), are another defined media preparations that require hydration and incubation for coliform and *E. coli*. These two products can be used in either the most-probable-number (MPN) or presence-absence (P-A) modes and have been evaluated in several studies (Edberg et al., 1988, 1989, Berger SA, 1991, Clark et al., 1991, and Olson et al., 1991). Another prepared medium, Petrifilm™ *E. coli* Count plates (3M Company, Minneapolis, MN), contains 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (BCIG) for GUS detection and selective agents found in VRB agar. This product enumerates coliforms as red colonies with gas bubbles, and *E. coli* as blue colonies with gas bubbles and has been tested with food samples (Matner et al., 1990). Whichever GUS detection system is used, however, a number of factors may influence the assay substantially. These include : strain differences in response to particular substrates and substrate concentration ; effects of carbohydrate content and selective agents in the medium ; incubation time and temperature ; pH changes ; ionic strength effects ; and possible

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interference by large numbers of competing bacteria or substances in the sample itself (Frampton et al., 1993). Methods based on detection of the enzyme GUS may not be entirely satisfactory. For example, Rice et al., 1990 reported that 97-99.5 % of *E. coli* produce GUS, and Chang et al., 1989 reported that basing a test on GUS activity may fail to detect a significant proportion (about 30%) of *E. coli* in some case because of occurrences of high incidences of GUS-negative *E. coli*. *E. coli* O157:H7 strains which have been linked to cases of hemorrhagic colitis and hemolytic uremic syndrome are also GUS-negative (Frampton et al., 1993).

6.5 *E. coli* detection by gene probes and polymerase chain reaction.

In less than a decade, interest in using nucleic acid probe technology for the detection of microorganisms has exploded. DNA hybridization techniques can be powerful tools in detection and identification of microorganisms from environment, crude samples, clinical material, water and food because they offer high sensitivity and specificity. DNA probe technology enables to screen specific DNA fragments of bacteria present in a sample ; detection and identification are performed without isolating the germ (Cleuziat et al., 1990). Because DNA probe detection is based on genotype rather than phenotype, DNA can be detected in a sample without assaying for cell growth or a protein product (Sayler et al., 1990). However, enhanced sensitivity is need to detect organisms present at low concentrations such as in water, or present only as a small percentage of the microbial community. Specific assays are also needed to detect the organism or gene sequence of interest in a background of other related organisms (Sayler et al., 1990). Improvements of special techniques to

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enhance the recovery of nucleic acids from samples which contain contaminating compounds is required to overcome such interference. The detection involves the pairing of target nucleic acid sequences with a homologous complementary probe sequence. Factors affecting the hybridization or reassociation of two complementary DNA strands include temperature, contacting time, salt concentration, the degree of mismatch between the base pairs, and the length and concentration of the target and probe sequences (Sayler et al., 1990).

Most current hybridization protocols are based on the hybridization of an immobilized target or probe molecule on a solid phase such as a nitrocellulose or nylon filter surface. The first step in filter hybridization is the attachment of single-stranded target nucleic acids to the filter surface. Next, the filters are prehybridized to block nonspecific nucleic acid binding sites. Labeled probe DNA is added to the filters and the probe is allowed to hybridize by re-establishing a double-stranded molecule with the complementary target sequences. After hybridization, excess unbound labeled probe is washed off and the hybrid (target:probe) sequences are detected (Sayler et al., 1990). Specificity is largely controlled by the stringency of the hybridization and washing condition. The advantages of filter hybridization are that different types of nucleic acid with varying purity can be analyzed (e.g. RNA, purified DNA, and unpurified DNA, and unpurified DNA from mixed bacterial colonies) and that multiple sample can easily be processed and quantified simultaneously (Sayler et al., 1990). Nucleic acid hybridization technology has been widely used to detect several organisms in various samples included various strains of *E. coli*. For example, Echeverria et al., 1982 detected ETEC in water by filter hybridization with three enterotoxin gene

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probes which carry the genes coding for the production of LT and ST and they reported that DNA hybridization assay to identifying ETEC in water was 10^4 times more sensitive than testing random *E. coli* in the Y-1 adrenal and suckling mouse assays. Boileau et al.,1984 used ^{32}P labeling, a 17-kilobase *Eco*RI restriction fragment of a virulence plasmid belonging to *Shigella frexneri* serotype 5 as a probe to detect *Shigella* species and enteroinvasive *E. coli* (EIEC) in isolated colonies. Begaud et al., 1993 reported the use of seven acetylaminofluorence (AAF)-labeled DNA probes in evaluating the incidence of various *E. coli* phatotypes included ETEC, EIEC and EPEC strains. There are also other similar studies which used DNA probe technique to detect *E. coli* phatotypes (Moseley et al., 1980, Venkatesan et al., 1988, and Willshaw G.A., 1992). The specific genetic marker of *E. coli* has also been studied by this technique. The DNA fragment contained part of *uidA* gene was tested as a DNA probe for specific detection of *E. coli* (Feng et al., 1991, Cleuziat et al., 1990 and Green et al., 1991). These studies reported that all *E. coli* and *Shigella* strains tested were reactive with the *uidA* gene probe, whether or not they were actively producing GUS. The detection of either *E. coli* or *Shigella* strains which are GUS-negative by *uidA* probe indicates that *uidA* gene is present but not expressed or produced a nonfunctional GUS. Genetic mutation in the regulatory or structural regions of the genome is suggested that such mutation can also affect *uidA* expression or the production of nonfunctional enzymes (Feng et al., 1991).

To improve detection sensitivity and specificity in bacterial detection, a new technology has been developed. The use of polymerase chain reaction (PCR) to amplify target DNA sequences is a new technology achieving rapid popularity. PCR is

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a technique for the in vitro amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA (Taylor GR., 1991). PCR also has many other potential uses, including DNA sequencing, cloning, and probe synthesis. The template for PCR can be ssDNA, dsDNA, or RNA (Sayler et al., 1990). The reaction is based on the annealing and extension of two oligonucleotide primers that flank the target region in duplex DNA ; after denaturation of the DNA, each primer hybridizes to one of the two separated strands such that extension from each 3′ hydroxyl end is directed toward the other. The annealed primers are then extended on the template strand with a DNA polymerase. These three steps (denaturation, primer binding, and DNA synthesis) represent a single PCR cycle. Consequently, repeated cycles of denaturation, primer annealing, and primer extension result in the exponential accumulation of a discrete fragment whose termini are defined by the 5′ ends of the primers. This exponential amplification results is obtained because under appropriate conditions the primer extension products synthesized in cycle "n" function as templates for the other primer in cycle "n+1" (Erlich et al., 1991). The key step to this procedure is the use of the thermostable *Thermus aquaticus* (*Taq*) DNA polymerase. This enzyme can function at temperatures up to 95°C and has greater fidelity than the *E. coli* DNA polymerase (Sayler et al., 1990). PCR has been used to detect microorganisms of environmental samples such as soil (Romanowski et al., 1993 and Picard et al., 1992), clinical samples such as sputum, cerebrospinal fluid, and stool (Kox et al., 1994, Greisen et al., 1994, Gillespie et al., 1994, and Schultsz et al., 1994), food samples (Bej et al., 1994, Kapperud et al., 1993, Gustafson et al., 1992, Wang et al., 1992., Lampel et al.,

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1990, and Giesendorf et al., 1992) and water samples (Oyofo et al., 1993, and Bej et al., 1991a, b, c). Application of the PCR to the identification of *E. coli* has been also studied (Bej et al., 1990, 1991a, b, c, and Cleuziat et al., 1990). PCR amplification of *lacZ* gene which encodes for β-galactosidase and *lamB* gene which encodes for a surface protein recognized by the *E. coli*-specific bacteriophage lambda was used to detect coliforms and *E. coli* (Bej et al., 1990). After amplification, radiolabeled gene probes are used for detecting genomic DNA of one to five viable cells of *E. coli* in 100 ml of water samples (Bej et al., 1990). The *uid* chromosomal region of *E. coli* including the *uidA* and its main regulatory gene, *uidR* were also tested as the target for *E. coli* detection (Bej et al., 1991a, b, c, Cleuziat et al., 1990). Cleuziat et al. reported the occurrence of 15% GUS-negative *E. coli* in their study and reported that the phenotypically GUS- negative *E. coli* could be detected by a PCR-gene probe test based upon the *uid* gene and sensitivity of this test was observed for at least eight bacteria per reaction. Bej et al., 1991a reported that PCR showed positive amplifications of both *uidA* and *uidR* targets for all *E. coli* tested and they were able to detect one to two viable cells of *E. coli* by using PCR and radiolabeled DNA probe for detection of *uidR* gene. Development of PCR amplification and gene probe detection technique can permit a rapid and reliable means of assessing the bacteriological safety of water and food and should provide an effective methodology to the conventional viable culture method. But PCR method is more sensitive and more rapid technique in detection of bacteria than DNA probe method.

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CHAPTER III

MATERIALS and METHODS

1. Microorganisms.

Most *E. coli*, other enteric bacteria and *Staphylococcus* used in this study were those isolated from patients, feeds, water and food samples. The 120 *E. coli* strains, including 11 Enteropathogenic *E. coli* (EPEC) strains, 14 Enterotoxigenic *E. coli* (ETEC) strains and other 85 nontoxigenic strains were obtained from Department of Pathology, Ramathibodi Hospital, Mahidol University, Enteric Bacteriology Section, Ministry of Public Health and from culture collection of BT 203, Department of Biotechnology, Faculty of Science, Mahidol University. The other 75 bacterial strains from genera of the family Enterobacteriaceae which composed of *Citrobacter*, *Enterobacter*, *Edwardsiella*, *Klebsiella*, *Morganella*, *Proteus*, *Salmonella*, *Shigella* and 2 strains of gram positive bacteria in genus *Staphylococcus* were also supplied by Department of Pathology, Ramathibodi Hospital, Mahidol University, Enteric Bacteriology Section and Salmonella-Shigella center (SS-center), Ministry of Public Health. *Pseudomonas putida* ATCC 12633 was kindly provided by Dr. Vithaya Meevootisom. Four *Bacillus* Strains used in this study were from culture collection of BT 203, Department of Biotechnology, Faculty of Science, Mahidol University. *E. coli* DH5-α, F^- f80d *lacZ*ΔM15Δ (*lacZYA-argF*) U19 *recA*1*endA*1 *hsdR* 17(r_k^--m_k^+) *sup* *E*44 1-*thi*-1 *gyrA*96 *relA*1, (BRL) was used as host and plasmid pGEM7(Ap^r) was used as vector in DNA transformation experiments. Plasmid pGA1

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and pGA2 containing a 0.153 kb and a 0.527 kb of *uid*A gene in pGEM7,respectively, were obtained from this study.

2. Culturing procedure.

All pure cultures of bacteria were kept as stock cultures by streaking on LB agar (1% tryptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar). The culture medium of *E. coli* DH5-α containing plasmids was suplemented with 50 μg/ml of ampicillin (Ap). For routine analysis, the culturers were grown on agar plates at 37^{o}C for overnight and were kept at 4^{o}C until used. For preservations, bacteria were grown until exponential phase in LB-broth on rotary shaker then 2 volumes of cell cultures were mixed with 1 volume of 45% glycerol and aliquots (1.2 ml) of the mixture were kept frozen at -70^{o}C in separated vials.

3. Chemicals and media.

All chemicals and reagents used in this study were analytical grade and obtained either from Merck (Germany), Sigma (USA), Fluka (Switzerland) or BRL (USA). Agarose, low melting agarose and Nusieve (3:1) agarose were purchased from BRL and FMC (USA). Restriction and modifying enzymes were obtained from either BRL, Biolab, Boehringer mannheim (Germany) or Pharmacia (Sweden). AmpliTaq DNA polymerase and PCR reaction buffer were obtained from Perkin-Elmer Cetus (USA). Ampicillin was bought from Sigma. Dig labeling and detection kit and

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deoxyribonucleoside triphosphate (dNTPs) were purchased from Boehringer Mannhiem. Two types of membrane filters, 0.45 µm pore size, 44-mm in diameter HA and 0.5 µm pore size, 13-mm in diameter Fluoropore FHLP membranes, were obtained from Millipore (USA). The GUS enzyme substrate 4-methylumbelliferyl β-D-glucuronide (MUG) was obtained from Biosynth AG (Switzerland) and other medium composition were obtained from Merck and Difco (USA).

Commercial Fluorocult ECD agar and Fluorocult Lauryl Sulfate broth were obtained from Merck (Germany). Synthetic Fluorocult plate count agar was prepared by mixing peptone (5 g), D(+) glucose(1 g), yeast extract (2.5 g), tryptophan (1 g) and agar (15 g) with 1 litre of distilled water and autoclaved. When the temperature of the medium was lowered down (about 55-60^{o}C), a 10 ml of filter sterilized MUG (0.007 g/ml) was added and mixed well. The medium was then dispensed into the wells of microtitration plates(150 µl/well) or poured into the petridish plates (20-25 ml/plate).

4. Preparation of DNA.

4.1 Chromosomal DNA preparation (large scale).

Chromosomal DNA of *E. coli* K12 and *E. coli* MM294 (F^- *endA*1 thi-1 *hsdR*17 *supE*44λ^-) were extracted by the procedure of Maniatis et al. (1989). The overnight bacterial culture in 5 ml of LB broth at 37^{o}C were inoculated to 200 ml of main culture and continuously incubated for 3 to 4 hrs. The bacteria were pelleted by centrifugation at 5,000 rpm for 5 min in RC-5 superspeed refrigerated centrifuge (Sorvall, Dupont, USA). The bacterial pellets were washed once with 25 mM Tris-

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HCl; pH 8.0 plus 5 mM EDTA then resuspended and incubated with 20 ml of same solution containing lysozyme 2-4 mg/ml for 30 min at 37°C. A 0.5 ml of 20% SDS was added and mixed immediately by inverting tube back and forth to prevent shearing until the solution became clear. Equal volume of saturated phenol was added, mixed gently and centrifuged at 8,000 rpm for 10 min. The aqueous phase which contained DNA was collected, and the procedure was repeated until white precipitate at the interphase was not seen. The aqueous phase was reextracted with phenol-chloroform-isoamyl alcohol (25:24:1) and then chloroform-isoamyl alcohol (24:1). DNA was precipitated with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of cold absolute ethanol and DNA was collected by winding around steriled stirring rod. The chromosomal DNA was washed with 70% ethanol, dried and dissolved in 1-2 ml of TE buffer (10 mM Tris-HCl; pH 7.5 and 1mM EDTA). DNA concentration was determined spectrophotometrically by measuring the optical density (OD) at 260nm and 280 nm. The value of OD260/OD280 should be around 2 which indicated purity of DNA. DNA concentration was calculated by using 1 OD unit is equal to 50 μg/ml. The chromosomal DNA was diluted to 100 ng/ml aliquoted and kept at -20°C until used.

4.2 Large scale plasmid preparation.

This method was modified from alkaline lysis method of Bimboim et al.(1979). Preculture was grown in 5 ml of LB broth supplemented with appropriate antibiotics (ampicillin 50 μg/ml) at 37°C overnight. The preculture was transferred to 250 ml of the same broth and incubated at 37°C with shaking for 10-15 hrs. The culture was

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centrifuged at 5,000 rpm for 5 min. The cell pellet was harvested and throughly suspended in 8 ml of solution I (25 mM Tris.HCl; pH 7.5, 10 mM EDTA, 15% sucrose and 2 mg/ml lysozyme), incubated on ice for 20 min. To lyse the cells, a 16 ml of solution II (0.2 N NaOH, 1 % SDS) was added to cell suspension, mixed and incubated on ice for 10 min. A 12 ml of solution III (3 M potassium acetate; pH5.2) was added, mixed and further maintained on ice for 10 min. The precipitate which contained cell debris, denatured DNA and protein were eliminated by centrifugation at 10,000 rpm for 10 min and the supernatant was filtered through the layer of cheese-cloth. The filtrate was added with 0.6 volume (21.6 ml) of isopropanol and centrifuged in order to precipitate DNA. The precipitate was dissolved in 10 ml of TE buffer, followed by addition with a 0.5 volume of 7.5 M ammonium acetate to precipitate RNA. The mixture was centrifuged at 10,000 rpm for 10 min to remove the precipitate. The remained RNA in supernatant was digested by treatment with RNase at final concentration 100-200 μg/ml at 65^{o}C for 10 min. The protein and other impurities were removed by extraction with phenol, phenol:chloroform and chloroform:isoamyl alcohol, respectively. DNA was precipitated with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of cold absolute ethanol and stored at -20^{o}C at least 30 min. The DNA was collected by centrifugation at 10,000 rpm for 10 min then washed once with 70% ethanol, dried and redissolved in 1-2 ml of TE buffer. The DNA concentration was determined by measuring OD_{260} or estimated on a 0.7% agarose gel with λ DNA as reference.

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4.3 Small scale plasmid preparation.

For small scale plasmid preparation, the STET miniprep of Holmes et al. (1981) method was used as a routine technique.

The bacteria was grown in 3-5 ml of LB-broth containing an appropriate antibiotic (ampicillin 50 µg/ml) at 37°C with shaking overnight. A 1.5 ml of cell culture was centrifuged at 5,000 rpm for 3 min. The bacterial cells were collected and resuspended in 500 µl of STET solution (8% sucrose, 5% triton X-100, 50 mM EDTA and 50 mM Tris-HCl; pH 8.0) then a 100 µl of STET solution containing 10 mg/ml lysozyme was added and mixed. The mixture was boiled for 2 min and then centrifuged at 10,000 rpm for 10 min to pack the slimy pellet which was removed by picking with toothpick. To precipitate the DNA, the supernatant was added with 0.7 volume of isopropanol, kept at -20°C for at least 30 min and then spun at 10,000 rpm for 10 min. The DNA pellet was washed with 70% ethanol, dried and resuspended in 50 µl of TE buffer.

4.4 Recovery of DNA fragment from agarose gel.

To obtain the PCR products or the desired DNA fragments, the DNA fragments were isolated from agarose gel (Maniatis et al.,1989). Agarose gel electrophoresis was performed with low temperature melting agarose gel. After the DNA bands were visualized under UV light, specific DNA band was cut from agarose gel by razor blade. The cut gel was transferred into an Eppendorf tube and one volume of TE buffer was added. The vial was incubated at 65°C for 10 min. Agarose was eliminated by extraction with saturated phenol. The aqueous phase was transferred into a new

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Eppendorf tube and reextracted with phenol:chloroform and chloroform:isoamyl alcohol respectively. Subsequently, the DNA was precipitated with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of absolute ethanol. The DNA was washed twice with 70% cold ethanol, dried and dissolved in TE buffer.

5. DNA primers and Polymerase Chain Reaction amplification.

5.1 DNA primers.

Three 22-mer primers used in this study were synthesized by Vetrogen Corporation (Ontario, Canada) and Bioservice Unit, Department of Biochemistry, Faculty of Science, Mahidol University (Bangkok, Thailand). All primers were derived from the coding region of *E. coli uidA* gene based on the sequence reported by Blanco et al. (1985) and Jefferson et al. (1986) (see Fig. 4). From the DNA sequence of the 2.439 kb insert of pRAJ 220 containing the β-glucuronidase gene of Jefferson, primer GAL-301 (5′-TGTTACGTCCTGTAGAAAGCCC-3′) was located between bps 301 and 322, primer GAR-432 (5′-AAAACTGCCTGGCACAGCAATT-3′) was located between bps 432 and 453 and primer GAR-806 (5′-GAGCAT TACGCTGCGATG GATC -3′) was located between bps 806 and 827. These primers were used to amplify two sizes of DNA fragments in the region of *uidA* gene. A 0.153 kb region of *uidA* gene was amplified with primer GAL-301 and GAR-432 and an extensive region, a 0.527 kb region of *uidA* gene, was amplified with primer GAL-301 and GAR-806. The positions of the three primers on map of a 1.806 kb *Hinf*I restriction sites on a whole *uidA* gene (Feng et al., 1991) were shown in Fig. 6.

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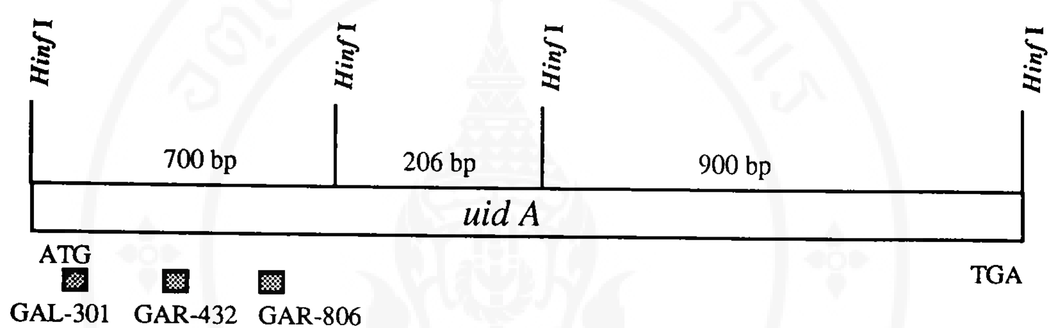

Fig . 6 Map of *Hinf*I restriction sites of *uidA* gene, showing position of primer GAL-301, GAR-432 and GAR-806. ATG and TGA are start and stop codons of *uidA* gene, respectively.

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               →
5′ 301 TGTTACGTCC TGTAGAAAGC CCCAACCCGT GAAATCAAAA AACTCGACGG CCTGTGGGCA 360
            Primer GAL-301

   361 TTCAGTCTGG ATCGCGAAAA CTGTGGAATT GATCAGCGTT GGTGGGAAAG CGCGTTACAA 420

   421 GAAAGCCGGG CAATTGCTGT GCCAGGCAGT TTTAACGATC AGTTCGCCGA TGCAGATATT 480
                 3′ TTAACGACA CGGTCCGTCA AAA 5′
                          Primer GAR-432      ←

   481 CGTAATTATG CGGGCAACGT CTGGTATCAG CGCGAAGTCT TTATACCGAA AGGTTGGGCA 540

   541 GGCCAGCGTA TCGTGCTGCG TTTCGATGCG GTCACTCATT ACGGCAAAGT GTGGGTCAAT 600

   601 AATCAGGAAG TGATGGAGCA TCAGGGCGGC TATACGCCAT TTGAAGCCGA TGTCACGCCG 660

   661 TATGTTATGT CCGGGAAAAG TGTACGTATC ACCGTTTGTG TGAACAACCA ACTGAACTGG 720

   721 CAGACTATCC CGCCGGGAAT GGTGATTACC GACGAAAACG GCAAGAAAAA GCAGTCTTAC 780

   781 TTCCATGATT TCTTTAACTA TGCCGGGATC CATCGCAGCG TAATGCTC 828 3′
                                3′ CTAG GTAGCGTCGC ATTACGAG 5′
                                        Primer GAR-806      ←
```

Fig. 7 DNA sequence of the 0.527 kp fragment containing some part of the *uidA* gene based on the sequence reported by Blanco et al., 1985 and Jefferson et al., 1983. Position of the 3 primers : primer GAL-301, primer GAR-432 and primer GAR-806 are underlined.

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DNA sequence of 0.527 kb containing some part of the *E. coli uidA* gene and the positions of the three primers were shown in Fig. 7. These primers were diluted with autoclaved deionized distilled water to reach a concentration of 20 µM woking solution and kept at -20°C until used.

5.2 PCR amplification from chromosomal DNA.

To amplify the portion of *uidA* gene, chromosomal DNA of *E. coli* K12 and *E. coli* MM294 extracted by the large scale procedure were used as DNA template. PCR amplification was performed in a 0.6 ml microcentrifuge tube as recommended in the instruction manual of GeneAmp PCR reagent kit with AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk Conn.). In a final volume of 100 µl, the reaction mixture contained 66.5 µl of autoclaved deionized distilled water, 10 µl of 10 x PCR reaction buffer (500 mM KCl, 100 mM Tris-HCl; pH 8.3, 0.01% [w/v] gelatin and 15 mM $MgCl_2$), 2 µl of 10 mM each of the deoxyribonucleoside triphosphate, dATP, dTTP, dCTP and dGTP, 0.5 µl of *Taq* DNA polymerase (5u/ml), 2.5 µl each of both primers and 10 µl of DNA template (100 ng/ml). The reaction vial was gently mixed, spun down in a microcentrifuge and then overlaid with 70 µl of autoclaved mineral oil to reduce evaporation. The final concentrations of each compositions were 50 mM KCl, 10mM Tris-HCl; pH 8.3, 0.001% [w/v]gelatin, 1.5 mM $MgCl_2$, 200 µM each of dNTP, 2.5 U of *Taq* DNA polymerase, 0.5 µM each of primers and 1 ng of DNA template. The mixture were processed in a programmable DNA thermal cycler (Robocycler 40, STRATAGENE). The program consisted of one cycle of 94°C for 3 min to ensure completely melting of the genomic DNA, then 30 cycles of

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denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. At the last cycle, the extension step at 72°C was performed for 5 min to complete partial polymerizations. The products were stored at 4°C.

5.3 PCR amplification from intact bacterial cells.

The specificity to amplify *uidA* gene directly from intact cells by PCR was performed according to the method of Joshi et al. (1991) with slight modification. All tested *E. coli* and other bacteria were grown in LB broth at 37°C with shaking overnight. One millilitre of each culture broth was transferred into an Eppendorf tube, heated at 100°C for 5 min and immediately cooled in ice water. These samples were then directly tested by PCR without isolation of the DNA. The reaction mixture, in a final volume of 100 µl, contained 50 mM KCl, 10 mM Tris-HCl; pH 8.3, 0.001% [w/v] gelatin, 1.5 mM $MgCl_2$, 200 µM each of dNTP, 2.5 U of *Tag* DNA polymerase, 0.5 µM each of primers, to which 10 µl of boiled cell lysate was added. After each sample was covered with 70 µl of mineral oil, the PCR was carried out in programable DNA thermal cycler. The PCR amplification was performed exactly as described earlier. Negative (containing water instead of template DNA) and positive (1 ng of purified genomic DNA from the *E. coli* K12) controls were also performed in each amplification experiment.

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6. Detection of amplified DNA.

After PCR amplification, aliquots of the reaction mixture were analysed. A 10-20 μl of PCR product was combined with 1-2 μl of loading buffer (50% sucrose, 50 mM Na_2EDTA, 0.05% bromphenol blue), and the preparation was electrophoresed on a 4% Nusieve 3:1 agarose gel or 1.5% agarose containing 0.5 μg/ml of ethidium bromide. Agarose gel was run in TBE buffer (0.045 M Tris-Borate, 0.001 M EDTA; pH 8.0) for 30 min at 100 V. Molecular size markers, *Pst*I-digested λ DNA, was included in the gel. After gel electrophoresis, the DNA was visualized by UV transilluminator (WL 300-310 nm). The UV illuminated gel was photographed with Polaroid FCR-10 at F/5.6, B1 and type 667 Polaroid film. If the PCR products were not analysed directly after the final cycle, the vials were kept at 4^oC until used. In some experiment, the detection of PCR products was performed by DNA hybridization. The technique will be described in sections 9 and 10.

7. Preparation of competent *E. coli* cells.

The competent *E. coli* were prepared by using the modified standard method (Hanahan et al., 1983 and Maniatis et al., 1989). A single colony of *E. coli* DH5-α was inoculated into 50 ml of Y-media (5 g/l yeast extract, 20 g/l tryptone, 5 g/l $MgSO_4.H_2O$ adjusted pH 7.6 with KOH and glacial acetic acid). The culture was incubated with shaking at 37^oC for 3-4 hrs. Preculture cells were transferred to 500 ml of another Y media (5% inoculation) and further incubated at 37^oC for 3-4 hrs

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until the culture reached the exponential phase (about 5-6 hrs). The culture was then chilled on ice for 5 min and cells were colleted by centrifugation at 3,000 rpm for 10 min at 4^oC. The supernatant was discarded and cells pellet was resuspended to 2/5 of original volume with filterred sterile TFB I solution (30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 $MnCl_2$, 15% v/v glycerol and adjusted pH 5.8 with 0.2 M acetic acid) and kept on ice for 5 min. The cells pellet was recovered by centrifugation at 3,000 rpm for 5 min at 4^oC and resuspended in 8 ml of the ice-cold filterred sterile TFB II solution (100 mM MOPS, 75 mM $CaCl_2.2H_2O$, 10 mM RbCl and 15% glycerol, adjusted pH to 6.5 with KOH). The competent cells were immediately frozen by immersing the Eppendorf tubes in liquid nitrogen and stored at -70^oC until used.

8. Cloning of PCR products.

8.1 Kinasing and Fill-in reaction of PCR products.

Kinasing and fill-in reaction of two sizes, 0.153, 0.527 kb, of PCR products were performed by the method of Kanungo et al. (1993). The amplified DNA was firstly purified by chloroform extraction to eliminate mineral oil. A 100 µl of chloroform was added after amplification. The mixture was vortexed and centrifuged at 10,000 rpm for 10 min. The aqueous phase containing the DNA which floated on the chloroform-oil mixture was collected and transferred to a new microcentrifuge tube. The DNA was precipitated and dissolved in TE buffer as described above. After precipitation, the 1-2 µg of DNA was subjected to kinase reaction. Kinase reaction mixture was a 40 µl solution contained 66 mM Tris-HCl; pH 7.6, 10 mM $MgCl_2$, 10 mM

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dithiothreitol, 0.2 mg/ml bovine serum albumin, 20 mM ATP and 2.0 units T_4 polynucleotide kinase. The mixture was incubated at 37^oC for 1 hr, then heated at 75^oC for 10 min to inactivate kinase activity and stored at 4^oC. Subsequently, the fill-in reaction was performed by adding 5 mM of deoxyribonucleoside triphosphates (dNTPs) and one unit of the Klenow fragment of DNA Polymerase I. The reaction mixture was incubated at room temperature for 30 min. The blunt ended DNA product was precipitated and dissolved in TE buffer.

8.2 Ligation of DNA fragments into vector.

The pGEM7 plasmid vector was linearized by digestion with *Ecol*CRI which generated blunt end fragments. *Ecol*CRI was the isochizomer of *Sac*I enzyme. The blunt end PCR products and the linearized pGEM7 plasmid vector were mixed together and warmed at 65^oC for 5 min then chilled on ice immediately. The ligation was performed, in a total volume of 15 µl, contained in a final concentration, 1 x ligation buffer (10 x ligation buffer composed of 200 mM Tris.HCl; pH 7.6, 50 mM $MgCl_2$, 50 mM dithiothreitol 500 µg/ml bovine serum albumin), 0.5 mM ATP and 10 Weiss units of T_4 DNA ligase. The reaction mixture was incubated at 4^oC overnight. The ligation product was used to transform into competent *E. coli* by the standard method of Maniatis et al. (1989).

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8.3 Transformation and selection.

The frozen competent cells were thawn in ice bath. A 3 μl of ligated product was added and mixed together. The mixture were stored on ice for 30 min. Then the mixture were heated at 42°C for 90 s and immediatly chilled on ice. Subsequently, 800 μl of LB broth was added, incubated at 37°C for 1 hr. The cultures were spreaded on LB agar containing ampicillin (50μg/ml) which was previously spreaded with 50 μl of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in formamide. The plates were incubated at 37°C overnight. The white colonies were picked and replica on LB agar containing 50 μg/ml ampicillin for further plasmid isolation and analysis. The white colonies which contained recombinant plasmids with either a 0.153 and a 0.527 kb DNA inserts as confirmed by restriction and gel eletrophoresis, were then picked. The plasmid from the 2 clones were designated pGA1 and pGA2, respectively.

9. DNA hybridization.

9.1 Preparation of digoxigenin-11-dUTP labeling DNA probes.

The plasmid pGA1 and pGA2 were digested by *Bam*HI and *Bst*XI to provide two sizes of DNA fragments, 0.175 and 0.549 kb, which contained the part of PCR products and some part of multicloning site (MCS) of vector pGEM7. These two fragments were recovered from gel and used as probes. In addition, the direct PCR product of a 0.153 and 0.527 kb fragments were also prepared as probes. After recovering from low temperature melting agarose gel by the method described

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previously, a 10 ng to 3 µg of these DNA was labeled with digoxigenin-11-dUTP by random primer method using DIG DNA labeling and detection kit (Boehringer Mannheim, Germany). The DNA was denatured for 10 min by heating in a boiling water bath and chilled quickly in ice/NaCl bath for 3 min, then hexanucleotide mixture, dNTP labeling mixture and 2 U Klenow enzyme were added and incubated at 37°C overnight. The reaction was stopped and precipitated with 0.2 M EDTA; pH 8.0, 4 M LiCl and 2 volumes of prechilled absolute ethanol. After incubation at 37°C for at least 1 hr, DIG -labeled DNA was obtained by centrifugation at 12,000 rpm for 10 min, then washed with 70% cold ethanol, dried and dissolved in 50 µl TE buffer. The labeled DNA probe was checked to confirm the successful labeling reaction with the detection step described below and the labeled probe was stored at -20°C until used.

9.2 Dot blot DNA-DNA hybridization.

The specificity of the *uidA* gene probes was determined by hybridization with various bacteria using dot blot hybridization technique. Bacterial cells were grown overnight with shaking at 37°C in LB-broth. A 100 µl of the culture was dotted onto nylon membrane using a vaccuum dot blot apparatus (Schleicher&Schull).

The DNA from cells deposited on membrane was released and denatured by placing the nylon membrane on Whatman paper No.1 saturated with lysing solution (0.5 M NaOH, 1.5 M NaCl) for 10 min and then the nylon membrane was removed and put on the other Whatman paper No.1 saturated with neutralizing solution (1M Tris-HCl; pH 7.0, 2 M NaCl) for 5 min and repeated neutralizing step with a fresh paper soaked in the same solution. The nylon membrane with bound DNA was

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washed in 2 x SSC (0.3 M NaCl, 0.03 M sodium citrate; pH 7.0). The DNA was fixed to the membrane by crosslinking with UV light (spectrolinker XL-1500, Spectronic Co; USA). The membrane can be used directly for hybridization or stored dry for later use in a seal plastic bag.

9.3 Prehybridization and hybridization.

The membrane was placed in a hybridization bag containing prehybridization solution (20 ml per 100 cm^2). The bag was sealed and prehybridized at 42°C for at least 1 hr. The solution for prehybridization consisted of 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 2.0% (w/v) blocking reagent (Boeringer Manheim), 0.1% N-laurylsarcosine, 0.02% (w/v) SDS and 50% (v/v) formamide. The hybridization solution was the same as prehybridization solution except that it contained denatured probe.

For hybridization, the DNA probe was denatured by heating in a boiling water bath for 10 min and immediately chilled in ice/NaCl bath for 3 min to prevent reannealing. The boiled labeled probe was added into pre-hybridization solution. At least 3.5 ml hybridization solution was used for membrane of 100 cm^2 in size. The prehybridization solution was discarded from the bag containing the membrane and the mixture of hybridization solution with heated denatured digoxigenin labeled DNA was replaced. The hybridization was performed at 42 °C overnight in a shaking water bath with constant shaking of the bag or can be done in hybridization oven.

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9.4 Detection of DIG-labeled probe-target hybrid.

After hybridization , the membrane was washed twice , 5 min per wash , with 2 x SSC/0.1% (w/v) SDS at room temperature and twice with 0.1 x SSC/0.1% SDS at 68°C for 15 min per wash with agitation. The hybridization signals could be detected directly, or the membrane was air-dried and stored for later detection. For detection, the membrane was equilibrated in buffer 1 (150 mM NaCl, 100 mM maleic acid; pH 7.5) for 1 min. The membrane was incubated with gently agitation at room temperature for 30 min in buffer 2 (1% [w/v] blocking reagent in buffer 1). Buffer 2 was decanted and the membrane was incubated in the antibody solution (anti-DIG-alkaline phosphatase 1:5000 in buffer 2) for 30 min at room temperature. Then, the unbound antibody-conjugate was removed by washing twice with buffer 1 for 15 min per wash. The membrane was rinsed with buffer 3 (100 mM Tris-HCl; pH 9.5, 100 mM NaCl and 50 mM $MgCl_2$) for 2 min. For colour development, the membrane was incubated in the dye solution containing 45 µl nitroblue tetrazolium (NBT) and 35 µl 5-bromo-4-chloro-3-indolylphosphate (x-phosphate) in 10 ml of buffer 3. The membrane was incubated in the dark. Once the desired spots or bands appeared, the reaction was stopped by washing the membrane for 5 min with buffer 4 (10 mM Tris-HCl, 1 mM EDTA; pH 8.0).

9.5 Southern blot DNA-DNA hybridization.

Southern blot DNA-DNA hybridization was performed to analyse the following DNA; the digested plasmid from various clones in tranformation experiment and the PCR amplified DNA from various bacteria.

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These DNA were electrophoresed in agarose gel containing 0.5 μg/ml EtBr. After taking photographs, DNA in gel were denatured by immersing in 0.5 M NaOH and 1.5 M NaCl for 30 min and then neutralized by soaking in 2 M NaCl, 1 M Tris-HCl; pH 7.0 for 30 min. The DNAs from agarose gel were transferred to nylon membrane by vacuum blotting technique (Milliblot-V Transfer System, Millipore Co; USA) for 1-1.5 hr. Then the membrane was baked by UV-crosslinking in order to fix the DNA to the membrane. The membrane was further pre-hybridized, hybridized and detected in the same procedure as described earlier.

10. Sensitivity determination of Dot blot hybridization and PCR technique for *E. coli* detection.

Sensitivity for *E. coli* detection was determined by two procedures, dot blot hybridization and PCR detection.

To determine the minimum number of cells per dot required to give a positive signal with the probes derived from *uidA* gene, *E. coli* K12 was grown overnight in LB-broth. The ten-fold serial dilutions in sterile 0.85% normal saline were made and dotted on nylon membrane and the membrane was hybridized as described in dot blot hybridization (9.2). The parallel plate count of the dilutions on MacConkey agar was simultaneously performed.

For sensitivity of PCR detection, ten fold dilution of overnight *E. coli* K12 culture were made in normal saline. Then one millilitre of each dilution was filtered through an absolute ethanol-presoked 13-mm Fluoropore membrane (FHLP; 0.5 μm

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pore size; Millipore Corp.) by using 5 ml sterile syringes and 13 mm swinnex disc filter holders then processed as described below in the procedure for detection of *E. coli* in water by using PCR (11.2). The parallel plate count of these dilutions was also performed. To confirm that PCR products was from *uidA*, the products were dotted on nylon membrane and further hybridized with the probes.

These methods were also used to determine the sensitivity for detection of *E. coli* in water and food by using the artificial contaminated sample. The artificial contaminated water and food samples were made by mixing 4.5 ml of *E. coli* free sample with 0.5 ml of ten fold serial dilutions of overnight grown *E. coli* K12 culture.

11. Detection of *E. coli* in water.

The drinking water samples from 9 different companies were purchased from the cafeteria of Mahidol University and Ramathibodi Hospital. These 9 samples were bottled in 500-950 ml plastic bottles. The other sample was collected from the drinking water tank of the Faculty of Science, Mahidol University. The positive and negative control were the autoclaved water artificially contaminated with *E. coli* K12 and the autoclaved water, respectively. The viable plate count of these water samples were determined by pour plate method (American Public Health Association, 1990) using synthetic fluorocult plate count agar with one millilitre of each undiluted and their ten-fold serial diluted samples. Detection of *E. coli* in these water samples was performed by using two procedures, membrane filter culture technique on Fluorocult ECD (*E. coli* direct) agar (Merck) and PCR detection.

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11.1 Membrane filter culture technique on Fluorocult ECD agar.

For membrane filter culture technique on Fluorocult ECD agar, 100 ml or five replicates of 20-ml portions of drinking water were filtered using filter holder through 47-mm-diameter, 0.45 µm-pore-size, type HA, membrane filters. This method was processed as essentially described in the standard membrane filter technique of American Public Health Association, 1987. Filters were then placed on Fluorocult ECD agar and incubated at 37°C for 24-48 hrs. After incubation, the plate was examined for the appearance of fluorescence under long-wave UV light (366nm). A blue fluorescence of the culture medium around the colonies indicated the presence of methlyumbelliferone (MU) which was converted from MUG by *uid*A product gene in *E. coli*. By dropping KOVACS' indole regent (Amyl or isoamyl alcohol 150 ml, Para-dimethylaminobenzaldehyde 10 g, HCl conc 50 ml) over individual colonies, the colour of reagent was changed to red which indicated the presence of indole. *E. coli* identification was positive when fluoresence was detected and the indole test was positive.

11.2 PCR detection.

The method for detection of *E. coli* in water using PCR was modified from the method of Bej et al. (1991c) and Wang et al. (1992). A 100 ml or five replicates of 20-ml portions of drinking water was filtered through an absolute ethanol-presoked 13-mm-diameter fluoropore membrane (FHLP; 0.5 µm pore size; Millipore Corp.) by using 20 ml sterile syringes and 13 mm swinnex disc filter holders. Each filter was picked and aseptically transferred with forceps to a 0.6 ml microcentrifuge tube with

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cell-coated side facing inwards. One hundred microlitres of autoclaved deionized distilled water was added to each tube which was vortexed vigorously for 5 to 10 s to release the cells from the filter surface to the liquid phase. DNA was released from bacterial cells by heating each tube at 100°C for 5 min in boiling water and immediately cooling in ice/NaCl bath. After heated-treatment process the samples were vortexed vigorously for 5 to 10 s to ensure the release of DNA from the surface of the filter. The samples were then spun for 2 to 3 s to collect any liquid on the wall or on the cap of the tube. The PCR reaction mix was added to each sample to a final volume of 150 µl and DNA amplification of the target DNA was performed without further purification. The PCR reaction mix consisted of 15 µl of 10 x reaction buffer (500 mM KCl, 100 mM Tris-HCl; pH8.3, 0.01% [w/v] gelatin and 15 mM $MgCl_2$) 12 µl of dNTPs mix (final concentration of 200 µM of each dNTP), 0.5 µM each primer and 5 U (1µl) of *Taq* DNA polymerase. Eighty microlitre of sterile mineral oil was added at the top of the sample. To prevent contact between the mineral oil and the filter, the edge of the filter was folded with a sterile neddle and submerged into the aqueous phase before adding the mineral oil. The target DNA was amplified in a DNA thermal cycler (Robocycler 40, STRATAGENE), using initial denaturation of the target DNA at 94°C for 3 min and PCR was performed as described previously. For detection of amplified DNA, 20 µl PCR-amplified DNA was used for gel eletrophoresis and about 120 µl was used for dot-blot DNA-DNA hybridization.

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12. Detection of *E. coli* in food sample.

Food sample tested was frozen boiled octopus which obtained from Food for Export Promotion Section, Division of Food for Export Analysis, Ministry of Public Health. To prepare food for *E. coli* detection, 25 g of the sample was mixed with 225 ml of LB-broth in sterile plastic bag. The mixture was homogenized with stomacher (Laboratory blender stomacher 400) for 1 min. So this homogenate was 1:10 dilution of food sample and then ten-fold dilution, 1:100, and 1:1,000, was performed. One millilitre of each ten-fold dilutions of the homogenate was used to determine the total microbial count by pour plate method with synthetic fluorocult plate count agar. For detection of *E. coli* in food sample, Multiple-tube Fermentation technique (AOAC,1990) with Fluorocult Lauryl Sulfate Broth (Merck) and PCR method were both performed.

12.1 Multiple-tube Fermentation technique with Fluorocult Lauryl Sulfate Broth.

Fluorocult Laural Sulfate Broth was prepared and suspened in 10 ml aliquot in test tubes which contained DURAHM tubes for gas observation. These tubes were arranged in a set of three tubes for testing each dilution of homogenized food sample. One millilitre of each ten fold dilution of homogenized food sample was inoculated into each set of 3 tubes. The inoculated tubes were mixed by gentle agitation and incubated at 37°C for 24-48 hrs. After 24 hrs, each tube was shaken gently and examined for gas production and appearance of fluorescence under UV light (366 nm). The presence of *E. coli* was indicated by the light blue fluorescence. If the result was

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negative, the tubes were further incubated and reexamined at 48 hrs after inoculation. To confirm the presence of *E. coli*, the light blue fluorescence tube was covered with KOVACS' reagent for about 5 mm in thickness. If the reagent layer became cherry red after 1-2 min, the presence of *E. coli* was confirmed.

12.2 PCR detection.

For detection of *E. coli* in food sample by using PCR technique, the original food sample and the artificial *E. coli* contaminated food sample (4.5 ml of *E. coli* free food homogenate mixed with 0.5 ml of one colony *E. coli* suspension in LB-broth) were tested. Homogenates of the two types of food samples were separated in 4 tubes. Each tube containing 5 ml of each sample were preenriched at 37°C for various incubation periods for 0h, 1h, 3h or 6h. The collected samples were analyzed by viable plate count, PCR detection and dot blot hybridization.

PCR detection was performed by two methods. The first method was processed by filtering one millilitre of the sample through an absolute ethanol-presoaked 13-mm-diameter fluoropore membrane (FHLP; 0.5 μm pore; MIllipore Corp.) using 5 ml sterile syringe and 13 mm swinnex disc filter. Then each filter with bacterial cells on it was used in the PCR assays by the procedure as described previously in the PCR detection of water samples (11.2). PCR products were subsequently detected by gel electrophoresis and dot blot hybridization.

The other method employed in detection of bacteria by PCR technique was performed by using 10 μl of boiled homogenate of food sample. This PCR method for

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detection of bacteria directly from food sample was carried out according to the method of PCR amplification from intact bacterial cells as described previously (5.3).

For analysis the possibility of PCR technique in detection of free DNA released from nonviable cells contaminated in food sample, the *E. coli* free food sample was inoculated with chromosomal DNA of *E. coli* K12. Homogenate of *E. coli* free food sample was aliquoted in 1 ml each and dispensed into 3 Eppendorf tubes and each tube was mixed with 1 ng of *E. coli* K12 chromosomal DNA. The mixtures were incubated in waterbath at 37°C for various periods. Each Eppendorf tube was collected either at 0, 30 or 60 min after incubation. The collected samples were centrifuged at 12,000 rpm for 10 min and the pellet was washed with autoclaved deionized distilled water. The mixture was resuspended in 100 µl of autoclaved deionized distilled water and mixed with 50 µl of PCR reaction mixture then subjected to PCR amplification. About 20 µl of PCR product was analysed in gel electrophoresis and about 120 µl was analysed in dot blot hybridization. The other experiment was performed in the similar way but filtration technique with FHLP membrane was performed instead of sample centrifugation.

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13. β-Glucuronidase microtiter plate assays.

The presence of β-glucuronidase produced by tested bacteria was determined by using synthetic Fluorocult plate count agar according to the method of Feng et al.(1982). The 150 μl of warm agar-substrate was transferred into the wells of microtiter plate. The overnight cultures of tested bacteria were stabbed into individual wells and covered with microtiter plate cover. The plate was incubated overnight at 37°C and then examined for the presence of fluorescence under long-wave UV light (366 nm).

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CHAPTER IV

RESULTS

1. Amplification of *uidA* gene portion from *E. coli* chromosome.

The *uidA* gene which encodes for enzyme β-glucuronidase is known to be quite specific for *E. coli* (Bej et al., 1991a, b, c, Cleuziat et al., 1990, Feng et al., 1991 and Green et al., 1991), therefore the gene sequence information may be used to detect *E. coli* contaminated in food or water. From sequence data of *uidA* gene reported by Blanco et al., 1985 and Jefferson et al., 1986, we selected 3 sequences GAL-301, GAR-432 and GAR-806 to be used as primers for detection of *E. coli* by PCR technique. The sequences of 3 primers were shown in Fig. 7 in which the primer positions are on the first half of the gene at 5′end (Fig.6). To obtain the DNA fragment which is specific for *E. coli* strains, the part of DNA sequences of the *uidA* gene was amplified by PCR method. PCR amplification was performed with the two pairs of oligonucleotide primers and chromosomal DNA of *E. coli* K-12 and *E. coli* MM294 were used as DNA templates. PCR system with the first pairs of primers, primers GAL-301 and GAR -432, generated the PCR fragments of 0.153 kb in length and the second pairs, primers GAL-301 and GAR-806 yielded the DNA fragment of 0.527 kb. The PCR products amplified from *E. coli* chromosome are shown in Fig. 8. Both *E. coli* K12 and MM294 gave the same size of PCR products when used the same pairs of primers (lane 2 and 4, lane 3 and 5).

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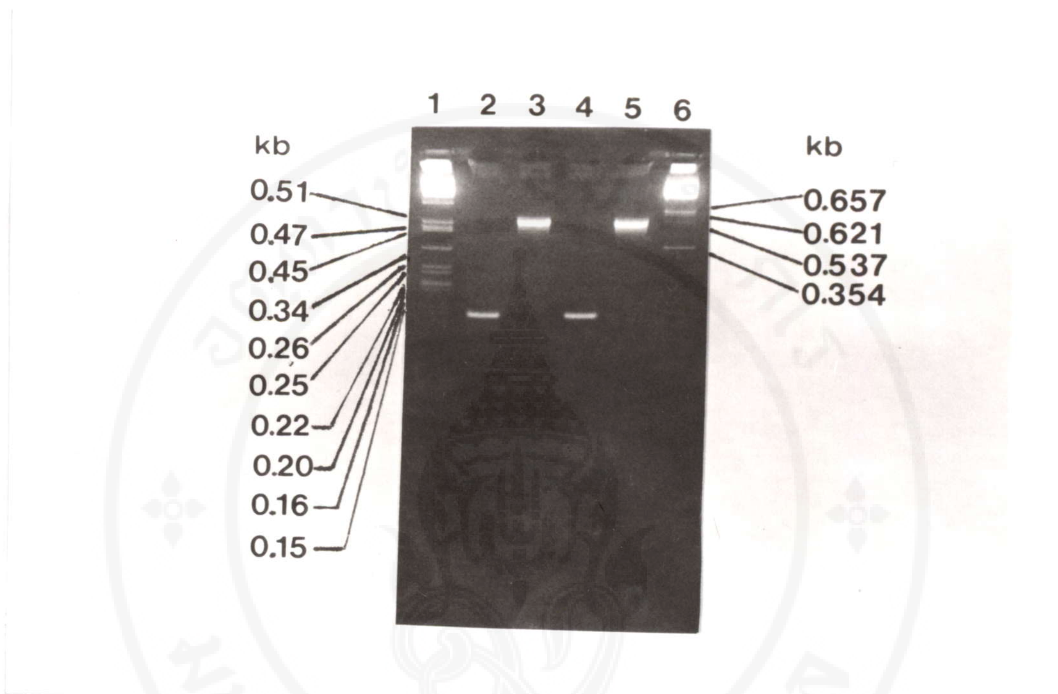

Fig. 8 Ethidium bromide-stained 4% Nusieve (3:1) agarose gel of PCR-amplified DNA from *uidA* gene of *E. coli* chromosome.

Lane 1. λ*Pst*I standard marker

Lane 2. *E. coli* K12 (GAL-301 and GAR-432 primers)

Lane 3. *E. coli* K12 (GAL-301 and GAR-806 primers)

Lane 4. *E. coli* MM294 (GAL-301 and GAR-432 primers)

Lane 5. *E. coli* MM294 (GAL-301 and GAR-806 primers)

Lane 6. λ*Hind*III standard marker

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2. Cloning of PCR products.

Construction of the clones harboring the PCR products which were the part of *uidA* gene was performed in order to be used as source of DNA probes. The two PCR products, a 0.153 and a 0.527 kb were purified by extraction from gel with saturated phenol and subjected to phosphorylation by kinase reaction and the end was made blunt by fill-in reaction with Klenow enzyme. The blunt end PCR products and the blunt end 3 kb pGEM7 vector (Fig. 9) which was linearized with *Ecol*CRI (isochisomer of *Sac*I enzyme) were ligated. The ligated products were transformed into *E. coli* DH5α and the transformants were selected on ampicillin and x-gal plates. The six white colonies of transformants were randomly selected and analyzed by digestion with *Bam*HI and *Bst*XI to check the size of inserted fragments. These digested plasmids provided two expected size of DNA fragments, a 0.175 kb (Fig. 10 lane 5-7, 10) and a 0.549 kb (Fig. 10 lane 8, 9). The extra 22 bp was from DNA sequence from the multicloning site obtained by *Bam*HI and *Bst*XI digestion. The recombinant plasmids which contained a 0.153 and a 0.527 kb inserted DNA fragments were designated as pGA1 and pGA2, respectively. These inserted DNA fragments were further confirmed by southern blot hybridization with digoxigenin labeled 0.153 and 0.527 kb PCR products probes. Fig. 11 and 12 showed agarose gel electrophoresis and southern blot hybridization results with the two probes. Since the 2 PCR products were from overlapped region therefore, both size of inserted DNA fragments gave the positive hybridization signals with these two probes (Fig. 11 and 12, lane 5-10). Both probes could also hybridize with the 2 size PCR products

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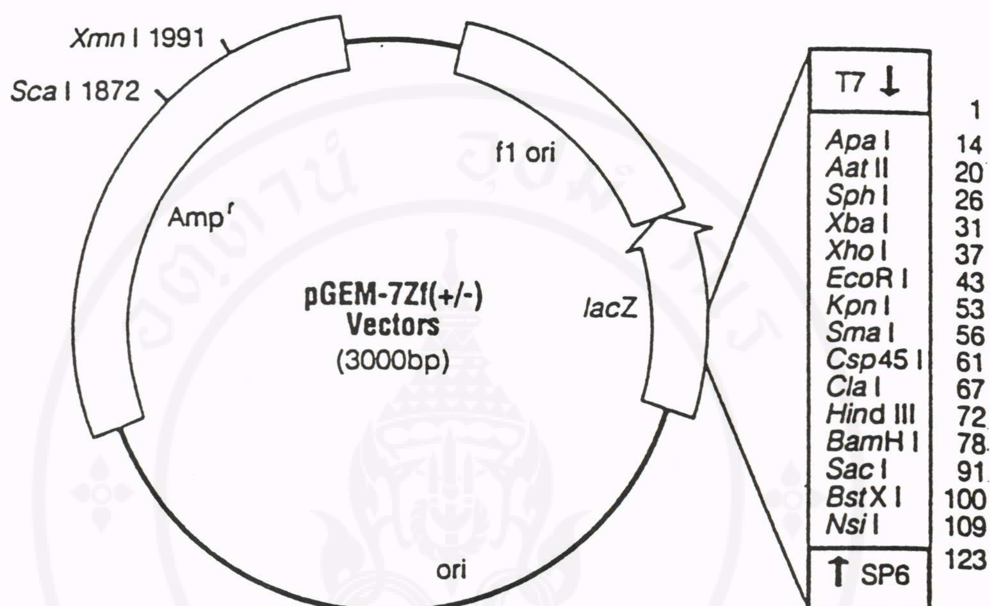

Fig. 9 The restriction map of pGEM-7Zf(+/-). The vector contains *E. coli* origin of DNA replication, T7 RNA polymerase promoter, SP6 RNA polymerase promoter, the multicloning site, lac operon sequence. The abbreviations in figure are as follows: Amp^r; ampicillin resistance gene, 1 ori; phage f1 region.

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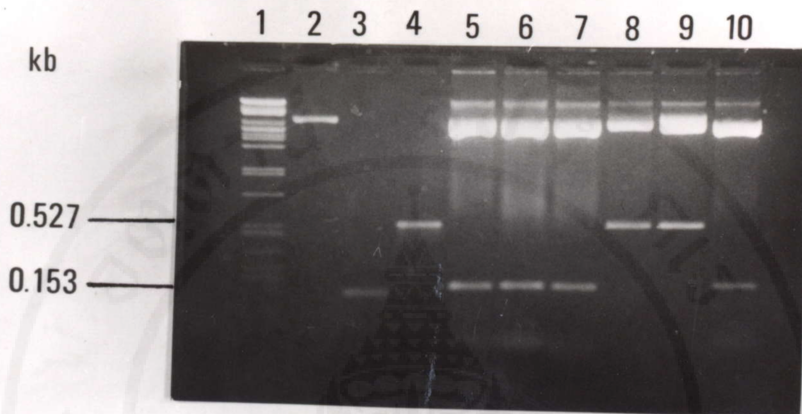

Fig. 10 Ethidium bromide-stained 1.5 % agarose gel of PCR products cloned in pGEM7. The plasmids from 6 transformants were cut with *Bam*HI and *Bst*XI.

Lane 1. λ*Pst*I standard marker

Lane 2. *EcoI*CRI-digested pGEM7

Lane 3. A 0.153 kb PCR products from *uidA* gene of *E. coli* K12

Lane 4. A 0.527 kb PCR products from *uidA* gene of *E. coli* K12

Lanes 5-7, 10. *Bam*HI and *Bst*XI digested plasmids from clones harboring a 0.175 kb insert.

Lanes 8-9. *Bam*HI and *Bst*XI digested plasmids from clones harboring a 0.549 kb insert.

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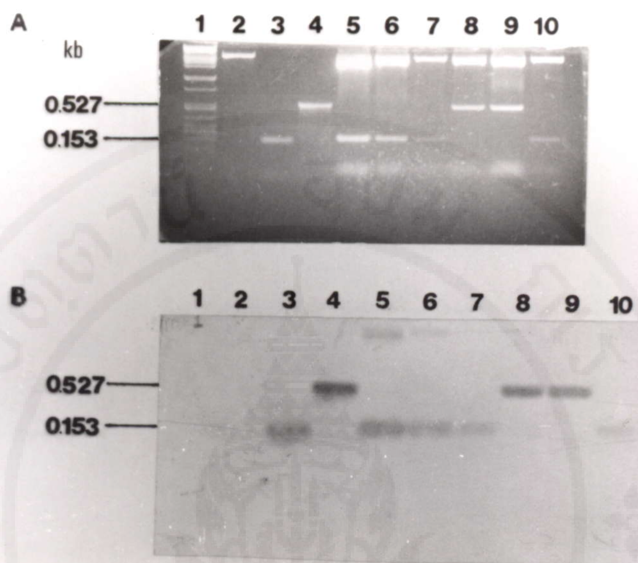

Fig. 11 Ethidium bromide-stained 1.5 % agarose gel (A) and Southern blot hybridization (B) using a 0.153 kb PCR products as probe to analyse the PCR products cloned in pGEM7 from various clones. The recombinant plasmids were cut with *Bam*HI and *Bst*XI.

Lane 1. λ*Pst*I standard marker

Lane 2. *Ecol*CRI-digested pGEM7

Lane 3. A 0.153 kb PCR products from *uidA* gene of *E. coli* K12

Lane 4. A 0.527 kb PCR products from *uidA* gene of *E. coli* K12

Lanes 5-7, 10. *Bam*HI and *Bst*XI digested plasmids from clones harboring a 0.175 kb insert.

Lanes 8-9. *Bam*HI and *Bst*XI digested plasmids from clones harboring a 0.549 kb insert.

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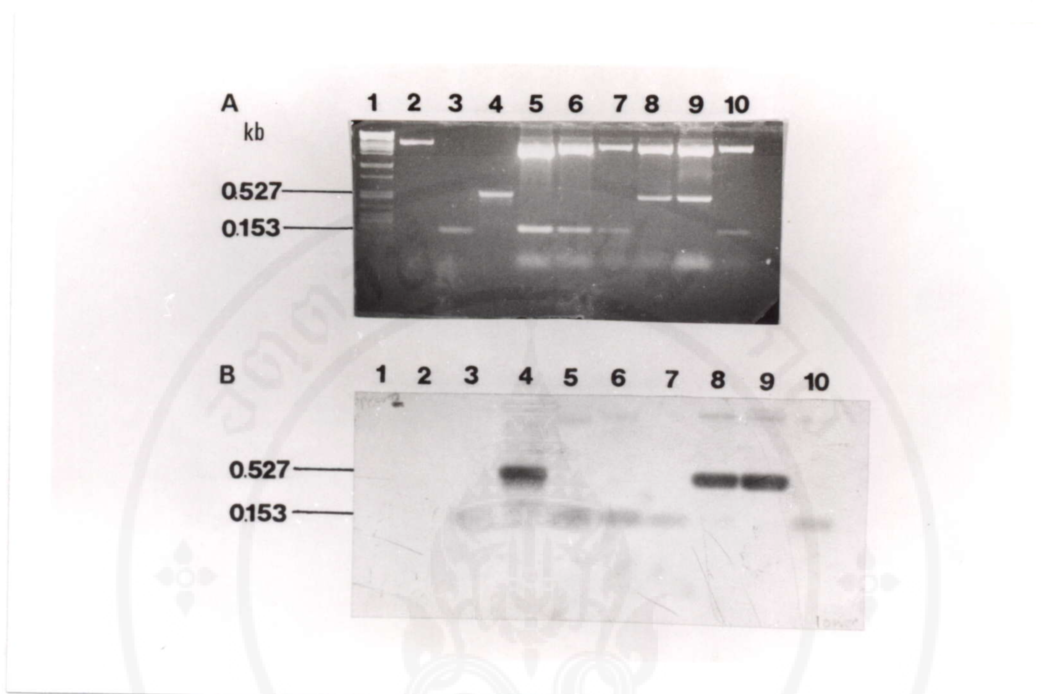

Fig. 12 Ethidium bromide-stained 1.5 % agarose gel (A) and Southern blot hybridization (B) using a 0.527 kb PCR products as probe to analyse the PCR products cloned in pGEM7 from various clones. The recombinant plasmids were cut with *Bam*HI and *Bst*XI.

Lane 1. λ*Pst*I standard marker

Lane 2. *Ecol*CRI-digested pGEM7

Lane 3. A 0.153 kb PCR products from *uidA* gene of *E. coli* K12

Lane 4. A 0.527 kb PCR products from *uidA* gene of *E. coli* K12

Lanes 5-7, 10. *Bam*HI and *Bst*XI digested plasmids from clones harboring a 0.175 kb insert.

Lanes 8-9. *Bam*HI and *Bst*XI digested plasmids from clones harboring a 0.549 kb insert.

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(Fig. 11 and 12 lane 3 and 4) but could not hybridize to pGEM7 (Fig. 11 and 12 lane 2). Therefore, it is concluded that both PCR products, a 0.153 and a 0.527 kb, were cloned in pGEM7 to obtain pGA1 and pGA2, respectively.

3. Detection of β-glucuronidase activity.

In order to compare the correlation between β-glucuronidase (GUS) expression and appearance of *uidA* gene in bacterial genome, all of 201 bacterial strains were examined for GUS activity by using microtiter plate assay. All bacterial strains tested were inoculated on the 150 μl of synthetic Fluorocult plate count agar poured in microtiter plate. The results were observed under UV light (366 nm). The presence of fluorescence indicated that methylumbelliferyl β-D-glucuronic acid (MUG) was converted to methylumbelliferone (MU) which is the fluorescent substance under long wave UV light. The results of GUS positive clones were shown in Table 4 and the example of fluorescence of some of them were shown in Fig. 13 and 14. The strains which could produce GUS enzyme displayed fluorescent which could be observed as bright colonies as shown in Fig. 13 Row A-D (except A7 and A8) and in Fig. 14 (A1, A3, A7, and A9-A11). The result from Table 5 can be summarized that among 201 strains tested, GUS activity was present in 96.8% of *E. coli* (91/94), 90.9% of enteropathogenic *E. coli* or EPEC (10/11), 100% of enterotoxigenic *E. coli* or ETEC (14/14) and 45.5% of *Shigella* spp. (5/11). All bacterial strains in other genera tested were GUS negative (see Table 5).

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Table 5 Bacterial strains tested for β-glucuronidase activity and hybridization results with probes GA153, GA527, GA175 and GA549

Microoganisms	β-glucuronidase detection[a]	Hybridization				
		probe GA153	probe GA527	probe GA175	probe GA549	position in Fig. 15, 16, 17, 18
1. *E.coli* B	+	+	+	+	+	A3
2. *E.coli* BHB 2688	+	+	+	+	+	A4
3. *E.coli* BHB 2690	+	+	+	+	+	A5
4. *E.coli* C600	+	+	+	+	+	A6
5. *E.coli* DH-1	-	+	+	+	+	B1
6. *E.coli* HB101	+	+	+	+	+	B2
7. *E.coli* IL9	+	+	+	+	+	B3
8. *E.coli* IL910	+	+	+	+	+	B4
9. *E.coli* JA221	+	+	+	+	+	B5
10. *E.coli* JM101	+	+	+	+	+	B6
11. *E.coli* JM103	+	+	+	+	+	C1
12. *E.coli* JM105	+	+	+	+	+	C2
13. *E.coli* JM109	+	+	+	+	+	C3
14. *E.coli* K12	+	+	+	+	+	A1, A7, G1, G7, M1, M7
15. *E.coli* MC1065	+	+	+	+	+	C4
16. *E.coli* MM294	+	+	+	+	+	C5
17. *E.coli* N113	+	+	+	+	+	C6
18. *E.coli* N131	+	+	+	+	+	D1
19. *E.coli* N133	+	+	+	+	+	D2
20. *E.coli* N135	+	+	+	+	+	D3
21. *E.coli* N137	+	+	+	+	+	D4
22. *E.coli* N1790	+	+	+	+	+	D5
23. *E.coli* PDC73	+	+	+	+	+	D6
24. *E.coli* RF53	+	+	+	+	+	E1
25. *E.coli* XL-1 blue	+	+	+	+	+	E2
26. *E.coli* 249	+	+	+	+	+	E3
27. *E.coli* 9484	+	+	+	+	+	E4
28. *E.coli* 2008/35	+	+	+	+	+	E5
29. *E.coli* 2010/35	+	+	+	+	+	E6
30. *E.coli* 2027/35	+	+	+	+	+	F1
31. *E.coli* 3516	+	+	+	+	+	F2
32. *E.coli* 3573	+	+	+	+	+	F3
33. *E.coli* 3589	+	+	+	+	+	F4
34. *E.coli* 3611	+	+	+	+	+	F5
35. *E.coli* 3612	+	+	+	+	+	F6
36. *E.coli* 3616	+	+	+	+	+	A9
37. *E.coli* 3618	+	+	+	+	+	A10
38. *E.coli* 3676	+	+	+	+	+	A11

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Table 5 Bacterial strains tested for β-glucuronidase activity and hybridization results with probes GA153, GA527, GA175 and GA549 (continued)

Microoganisms	β-glucuronidase detection[a]	Hybridization				
		probe GA153	probe GA527	probe GA175	probe GA549	position in Fig. 15, 16, 17, 18
39. *E.coli* 3678	+	+	+	+	+	A12
40. *E.coli* 3689	+	+	+	+	+	B7
41. *E.coli* 3686	+	+	+	+	+	B8
42. *E.coli* 3725	+	+	+	+	+	B9
43. *E.coli* 3731	+	+	+	+	+	B10
44. *E.coli* 3785	+	+	+	+	+	B11
45. *E.coli* 3801	+	+	+	+	+	B12
46. *E.coli* 3815	+	+	+	+	+	C7
47. *E.coli* 129/36	+	+	+	+	+	C8
48. *E.coli* 132/36	+	+	+	+	+	C9
49. *E.coli* 133/36	+	+	+	+	+	C10
50. *E.coli* 134/36	+	+	+	+	+	C11
51. *E.coli* 135/36	+	+	+	+	+	C12
52. *E.coli* 136/36	+	+	+	+	+	D7
53. *E.coli* 137/36	+	+	+	+	+	D8
54. *E.coli* 148/36	+	+	+	+	+	D9
55. *E.coli* 149/36	+	+	+	+	+	D10
56. *E.coli* 150/36	+	+	+	+	+	D11
57. *E.coli* 151/36[b]	-	+	+	+	+	D12
58. *E.coli* 152/36[b]	+	+	+	+	+	E7
59. *E.coli* 153/36	+	+	+	+	+	E8
60. *E.coli* 154/36	+	+	+	+	+	E9
61. *E.coli* 155/36	+	+	+	+	+	E10
62. *E.coli* 156/36	+	+	+	+	+	E11
63. *E.coli* 157/36	+	+	+	+	+	E12
64. *E.coli* 163/36	+	+	+	+	+	F7
65. *E.coli* 164/36	+	+	+	+	+	F8
66. *E.coli* 165/36	+	+	+	+	+	F9
67. *E.coli* 166/36	+	+	+	+	+	F10
68. *E.coli* 168/36	+	+	+	+	+	F11
69. *E.coli* 169/36	+	+	+	+	+	F12
70. *E.coli* 170/36	+	+	+	+	+	G3
71. *E.coli* 171/36	+	+	+	+	+	G4
72. *E.coli* 172/36	+	+	+	+	+	G5
73. *E.coli* 173/36	+	+	+	+	+	G6
74. *E.coli* 174/36	+	+	+	+	+	H1
75. *E.coli* 175/36	+	+	+	+	+	H2
76. *E.coli* 176/36	+	+	+	+	+	H3

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Table 5 Bacterial strains tested for β-glucuronidase activity and hybridization results with probes GA153, GA527, GA175 and GA549 (continued)

Microoganisms	β-glucuronidase detection[a]	Hybridization				
		probe GA153	probe GA527	probe GA175	probe GA549	position in Fig. 15, 16, 17, 18
77. *E.coli* 177/36	+	+	+	+	+	H4
78. *E.coli* 178/36	+	+	+	+	+	H5
79. *E.coli* 179/36	-	+	+	+	+	H6
80. *E.coli* 180/36	-	+	+	+	+	I1
81. *E.coli* 181/36	+	+	+	+	+	I2
82. *E.coli* 182/36	+	+	+	+	+	I3
83. *E.coli* 183/36	+	+	+	+	+	I4
84. *E.coli* 184/36	+	+	+	+	+	I5
85. *E.coli* 185/36	+	+	+	+	+	I6
86. *E.coli* 186/36	+	+	+	+	+	J1
87. *E.coli* 187/36	+	+	+	+	+	J2
88. *E.coli* 188/36	+	+	+	+	+	J3
89. *E.coli* 189/36	+	+	+	+	+	J4
90. *E.coli* 190/36	+	+	+	+	+	J5
91. *E.coli* 191/36	+	+	+	+	+	J6
92. *E.coli* 195/36	+	+	+	+	+	K1
93. *E.coli* 197/36	+	+	+	+	+	K2
94. *E.coli* 198/36	+	+	+	+	+	K3
95. *E.coli* 199/36	+	+	+	+	+	K4
96. *E.coli* 200/36	+	+	+	+	+	K5
97. *E.coli* 201/36	+	+	+	+	+	H6
98. *E.coli* 202/36	+	+	+	+	+	L1
99. *E.coli* 203/36	+	+	+	+	+	L2
100.*E.coli* 207/36[b]	+	+	+	+	+	L3
101.*E.coli* 213/36	+	+	+	+	+	L4
102.*E.coli* 216/36	+	+	+	+	+	L5
103.*E.coli* 217/36	+	+	+	+	+	L6
104.*E.coli* 220/36	+	+	+	+	+	M9
105.*E.coli* 221/36	+	+	+	+	+	M10
106.*E.coli* 222/36[b]	+	+	+	+	+	M11
107.*E.coli* 51/35[c]	+	+	+	+	+	M12
108.*E.coli* 105/35[c]	+	+	+	+	+	N7
109.*E.coli* 157/35[c]	+	+	+	+	+	N8
110.*E.coli* 265/35[c]	+	+	+	+	+	N9
111.*E.coli* 569/35[c]	+	+	+	+	+	N10
112.*E.coli* 897/35[c]	+	+	+	+	+	N11
113.*E.coli* 939/35[c]	+	+	+	+	+	N12
114.*E.coli* 1051/35[c]	+	+	+	+	+	O7

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Table 5 Bacterial strains tested for β-glucuronidase activity and hybridization results with probes GA153, GA527, GA175 and GA549 (continued)

Microoganisms	β-glucuronidase detection[a]	Hybridization				
		probe GA153	probe GA527	probe GA175	probe GA549	position in Fig. 15, 16, 17, 18
115.*E.coli* 1054/35[c]	+	+	+	+	+	O8
116.*E.coli* 1636/35[c]	+	+	+	+	+	O9
117.*E.coli* 1644/35[c]	+	+	+	+	+	O10
118.*E.coli* 1690/35[c]	+	+	+	+	+	O11
119.*E.coli* 1692/35[c]	+	+	+	+	+	O12
120.*E.coli* 1741/35[c]	+	+	+	+	+	P7
121.*Citrobacter freundi*	-	-	-	-	-	I8
122.*C.freundi* GN 810/37	-	-	-	-	-	P8
123.*C.diversus* GN 834/37	-	-	-	-	-	P9
124.*C.*spp.3548	-	-	-	-	-	I9
125.*C.*spp.3680	-	-	-	-	-	I10
126.*Enterobacter aerugenes* DM1333	-	-	-	-	-	P10
127.*E.cloacae*	-	-	-	-	-	I11
128.*E.cloacae* 3573	-	-	-	-	-	I12
129.*E.cloacae* 3589	-	-	-	-	-	J7
130.*E.cloacae* GN 837/37	-	-	-	-	-	O11
131.*Edwardsiella tarda*	-	-	-	-	-	J8
132.*Klebsiella ozaenae* YN867/37	-	-	-	-	-	P12
133.*K.pneumoniae*	-	-	-	-	-	J9
134.*K.pneumoniae* 3612	-	-	-	-	-	J10
135.*K.pneumoniae* 3618	-	-	-	-	-	J11
136.*K.pneumoniae* 3725	-	-	-	-	-	J12
137.*K.pneumoniae* 3859	-	-	-	-	-	K7
138.*K.pneumoniae* GN 834/37	-	-	-	-	-	Q7
139.*Morganella morganii*	-	-	-	-	-	K8
140.*Proteus vulgalis*	-	-	-	-	-	K9
141.*Pseudomonas putida* 12633	-	-	-	-	-	A2,A8,G2 G8,M2,M8
142.*Salmonella* Aberdeen	-	-	-	-	-	K10
143.*S.*Agona	-	-	-	-	-	K11
144.*S.*Alachua	-	-	-	-	-	K12
145.*S.*Amsterdam	-	-	-	-	-	L7
146.*S.*Anatum 1728	-	-	-	-	-	L8
147.*S.*Anatum 1729	-	-	-	-	-	L9
148.S.Berta	-	-	-	-	-	L10
149.*S.*Bovis	-	-	-	-	-	L11

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Table 5 Bacterial strains tested for β-glucuronidase activity and hybridization results with probes GA153, GA527, GA175 and GA549 (continued)

Microoganisms	β-glucuronidase detection[a]	Hybridization				
		probe GA153	probe GA527	probe GA175	probe GA549	position in Fig. 15, 16, 17, 18
150.*S*.Breadency	-	-	-	-	-	L12
151.*S*.Cerro 414	-	-	-	-	-	M3
152.*S*.Choleraesuis	-	-	-	-	-	M4
153.*S*.Derby	-	-	-	-	-	M5
154.*S*.Dublin	-	-	-	-	-	M6
155.*S*.Emek 174	-	-	-	-	-	N1
156.*S*.Emek 9161	-	-	-	-	-	N2
157.*S*.Enteritidis1781	-	-	-	-	-	N3
158.*S*.Enteritidis1890	-	-	-	-	-	N4
159.*S*.Falkensee	-	-	-	-	-	N5
160.*S*.Gera	-	-	-	-	-	N6
161.*S*.Havana 368	-	-	-	-	-	O1
162.*S*.Heidelberg	-	-	-	-	-	O2
163.*S*.Hwittingfoss	-	-	-	-	-	O3
164.*S*.Infantis 1160	-	-	-	-	-	O4
165.*S*.Isangi	-	-	-	-	-	O5
166.*S*.Krefeld 1712	-	-	-	-	-	O6
167.*S*.Krefeld 1735	-	-	-	-	-	P1
168.*S*.Langensalza	-	-	-	-	-	P2
169.*S*.Lexington1788	-	-	-	-	-	P3
170.*S*.Livingstone	-	-	-	-	-	P4
171.*S*.Mbandaka	-	-	-	-	-	P5
172.*S*.Mbandaka 1844	-	-	-	-	-	P6
173.*S*.Mbandaka 1845	-	-	-	-	-	Q1
174.*S*.Monterideo	-	-	-	-	-	Q2
175.*S*.Muenchen	-	-	-	-	-	Q3
176.*S*.Ohio 660	-	-	-	-	-	Q4
177.*S*.Paratyphi A	-	-	-	-	-	Q5
178.*S*.Panama 832	-	-	-	-	-	Q6
179.*S*.Senftenberg	-	-	-	-	-	R1
180.*S*.Saintpaul/ 229	-	-	-	-	-	R2
181.*S*.Typhimurium	-	-	-	-	-	R3
182.*S*.Urbana 1169	-	-	-	-	-	R4
183.*S*.Virchow	-	-	-	-	-	R5
184.*S*.Worthington	-	-	-	-	-	R6
185.*Shigella boydii*	-	+	+	+	+	G9
186.*S.flexneri*	+	+	+	+	+	G10
187.*S.flexneri* 2a	-	+	+	+	+	G11

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Table 5 Bacterial strains tested for β-glucuronidase activity and hybridization results with probes GA153, GA527, GA175 and GA549 (continued)

Microoganisms	β-glucuronidase detection[a]	Hybridization				
		probe GA153	probe GA527	probe GA175	probe GA549	position in Fig. 15, 16, 17, 18
188.*S.flexneri* 3a	-	+	+	+	+	G12
189.*S.flexneri* 2b	-	+	+	+	+	H7
190.*S.flexneri* 6	+	+	+	+	+	H8
191.*S.flexneri* varx	-	+	+	+	+	H9
192.*S.sonnei* I	+	+	+	+	+	H10
193.*S.sonnei* I, II	+	+	+	+	+	H11
194.*S.sonnei* 781	+	+	+	+	+	H12
195.*S.sonnei* 3310	-	+	+	+	+	I7
196.*Bacillus megaterium*	-	-	-	-	-	Q8
197.*B.sphericus*	-	-	-	-	-	Q9
198.*B.subtilis*	-	-	-	-	-	Q10
199.*B.thuringiensis*	-	-	-	-	-	Q11
200.*Staphylococcus aureus* 980	-	-	-	-	-	Q12
201.*S.aureus* 10442	-	-	-	-	-	R7

[a] The GUS detection was performed by suspending colony of *E.coli* in 150 μl of MUG (0.007 g/ml) and observed fluorescence under long wave UV light (366 nm).

[b] Enteropathogenic *E.coli*.

[c] Enteropathogenic *E.coli*.

\+ β glucuronidase positive or hybridization signal positive.

\- β glucuronidase negative or hybridization signal negative.

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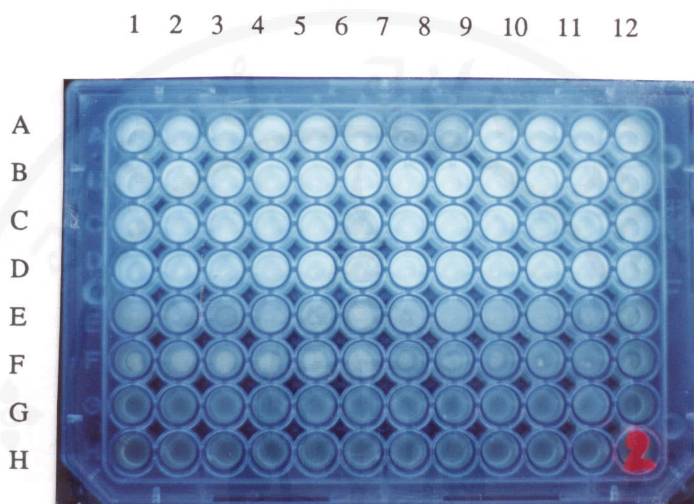

Fig. 13 β-glucuronidase microtiter plate assay. Row A-D (left to right) contain *E. coli* No. 73-120 listed in Table 5. Row E-F contain other bacteria No.121-144 listed in Table 5. Row G-H contain no bacteria. All *E. coli* display fluorescence except *E. coli* No. 79 and 80 which are *E. coli* 179/36 (A7) and *E. coli* 80/36 (A8) respectively, give negative signals. Other bacteria than *E. coli* and *Shigella* in Row E-F also show negative results.

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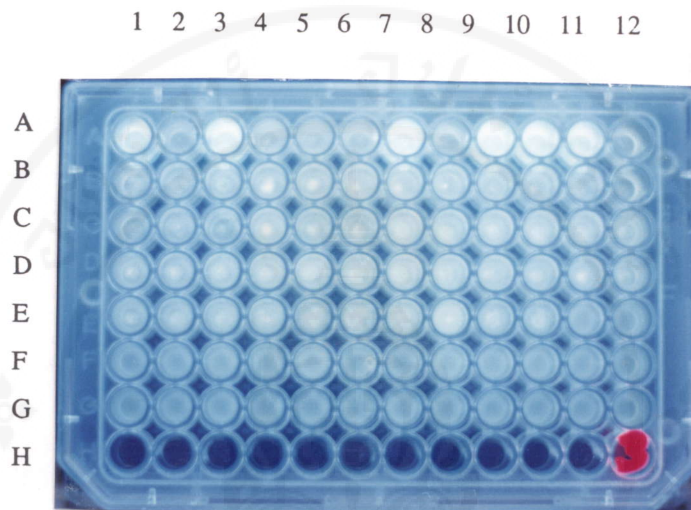

Fig. 14 β-glucuronidase microtiter plate assay. A1 is *E. coli* K12 and the other wells in row A (left to right) are *Shigella* spp. No. 185-195 listed in Table 5. Row B-E except E11 and E12 contain other bacteria from No.145-184 and No.196-201 as listed in Table 5. Row F-G and E11-E12 contain no bacteria. All wells of row H are empty wells. *E. coli* K12 (A1), *Shigella flexneri* (A3), *S sonnei* I (A9), *S. sonnei* I, II (A10) and *S. sonnei* 781 (A11) give positive signals, whereas other bacteria show negative results.

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4. Specificity test for detection of *E. coli*.

Specificity test for detection of *E. coli* was performed by two procedures which both were based on *uidA* gene. The first procedure was determined by dot blot hybridization using GA153, GA527, GA175, and GA549 probes. The second procedure was performed by PCR amplification from intact bacterial cells using primers derived from *uidA* gene. Various *E. coli* strains as well as other gram positive and gram negative bacteria were used for both dot blot hybridization and PCR techniques.

4.1 Specificity of *uidA* probes.

Dig-labeled DNA probes of four DNA fragments which located on the coding region of *uidA* gene were prepared. These DNA fragments were a 0.153 kb and a 0.527 kb PCR products, a 0.175 kb and a 0.549 kb *Bam*HI/*Bst*XI restriction fragments from plasmids pGA1 and pGA2. They were designated as probes GA153, GA527, GA175 and GA549, respectively. The probes GA175 and GA549 were derived from GA153 and GA527, respectively as a result of subcloning of GA153 and GA527 in pGEM7 to obtain plasmids pGA1 and pGA2. Cutting of pGA1 and pGA2 with *Bam*HI/*Bst*XI added the extra 22 bp from multicloning site to GA153 and GA527. Specificity test of these probes in bacterial detection were performed by dot blot hybridization with 120 strains of *E. coli* which included 11 strains of Enteropathogenic *E. coli* (EPEC) and 14 strains of Enterotoxigenic *E. coli* (ETEC). Seventy five strains of other *Enterobacteriaceae* and 6 strains of gram positive bacteria were also

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included in hybridization experiments. The 100 μl of overnight broth culture of these bacteria were dotted on nylon membrane by using dot blot apparatus. The membrane with bound cells was denatured and neutralized before hybridization with the probes. The list of bacteria, hybridization results and β-glucuronidase assay were shown in Table 5. The hybridization signal on nylon membrane of these strains to these four probes were shown in Fig. 15, 16, 17 and 18. The results of hybridization obtained with these four probes gave no difference observation. Therefore the extra 22 bp from the multicloning site of pGEM7 in probes GA175 and GA549 did not cause any cross hybridization. All strains of *E. coli* (100%) and *Shigella* spp. (100%) could hybridize with these probes even though 5 and 6 strains of *E. coli* and *Shigella*, respectively did not produce GUS (see Table 5). No hybridization signal and enzyme GUS activity were observed among other entericbacteria (except some strains of *Shigella* produced GUS enzyme) and gram positive bacteria (Table 5).

4.2 Specificity of PCR primers

To determine the specificity of the DNA amplification with two sets of primers , intact bacterial cells from strains tested in this study were subjected directly to PCR amplification. PCR amplification was performed in a programmable DNA thermal cycler (Robocycler 40, STRATAGENE). The optimum program consisted of one cycle of 94°C for 3 min, then 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and the last cycle was performed at 72°C for 5 min.

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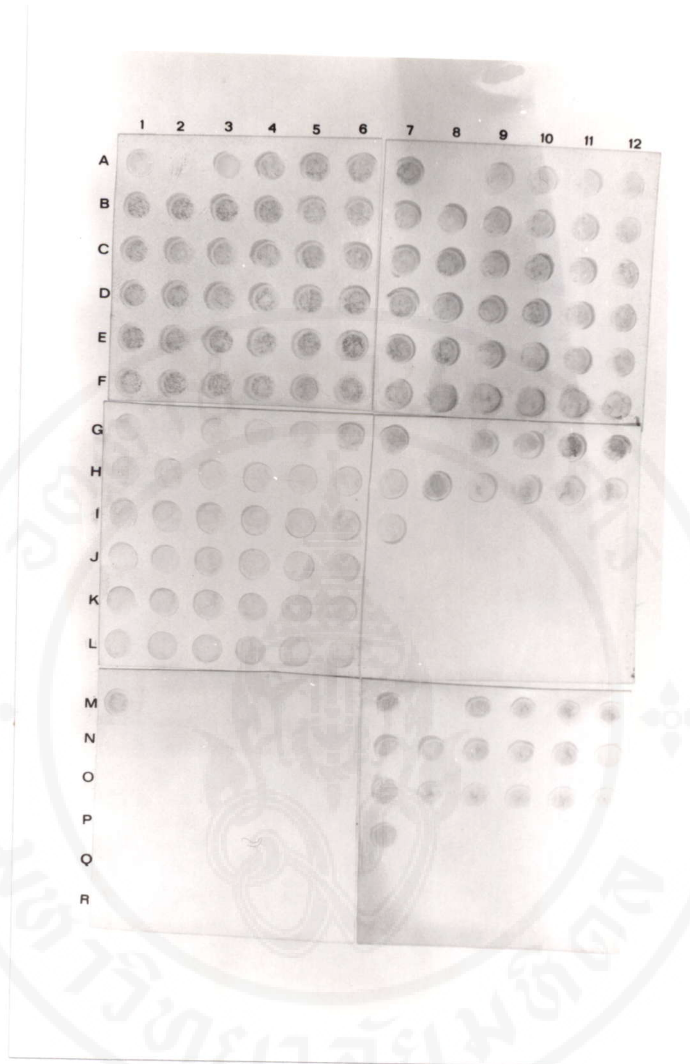

Fig. 15 Dot blot hybridization of *E. coli* and other bacteria with GA153 probe. All positive signals are *E. coli* strains except G9-G12, H7-H12 and I7 are *Shigella* spp. A2, A8, G2, G8, M2 and M8 are *Pseudomonas putida*. The other negative signal positions are other enteric bacteria or other gram positive bacteria as indicated in Table 5. R8-R12 are blanks.

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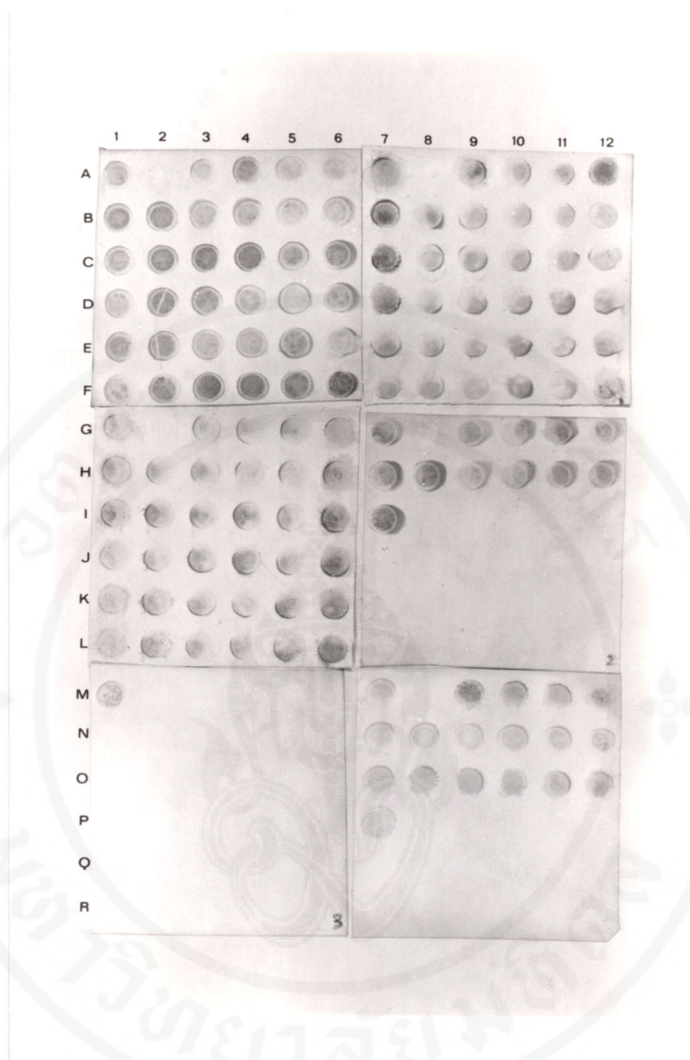

Fig. 16 Dot blot hybridization of *E. coli* and other bacteria with GA527 probe. The types of bacteria and positions on the membrane are the same as described in Fig. 15.

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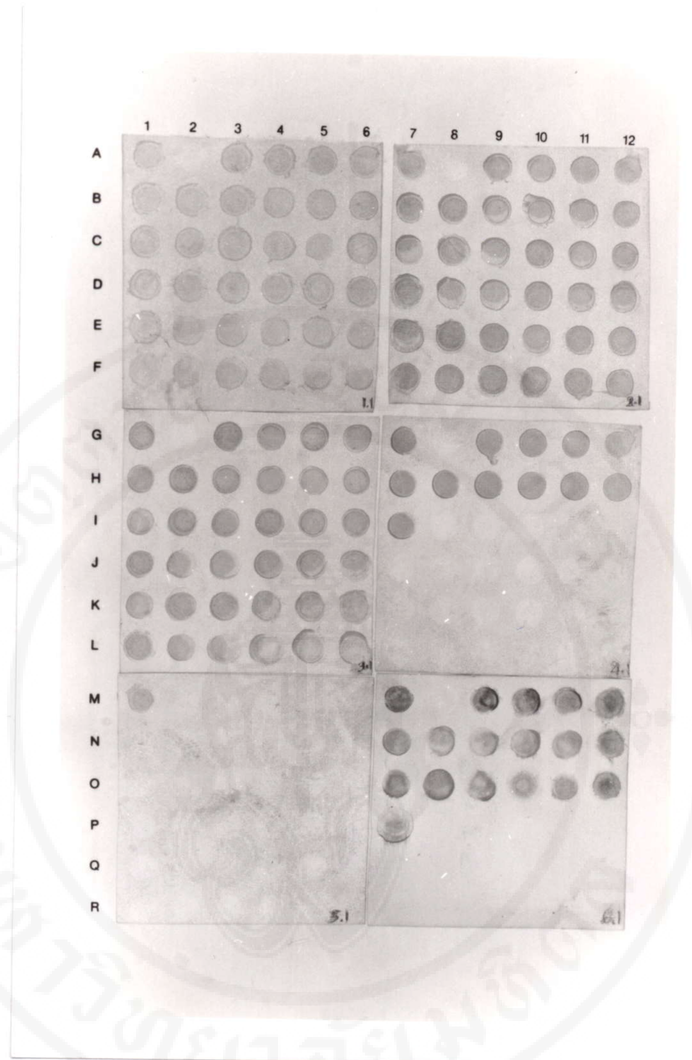

Fig. 17 Dot blot hybridization of *E. coli* and other bacteria with GA175 probe. The types of bacteria and positions on the membrane are the same as described in Fig. 15.

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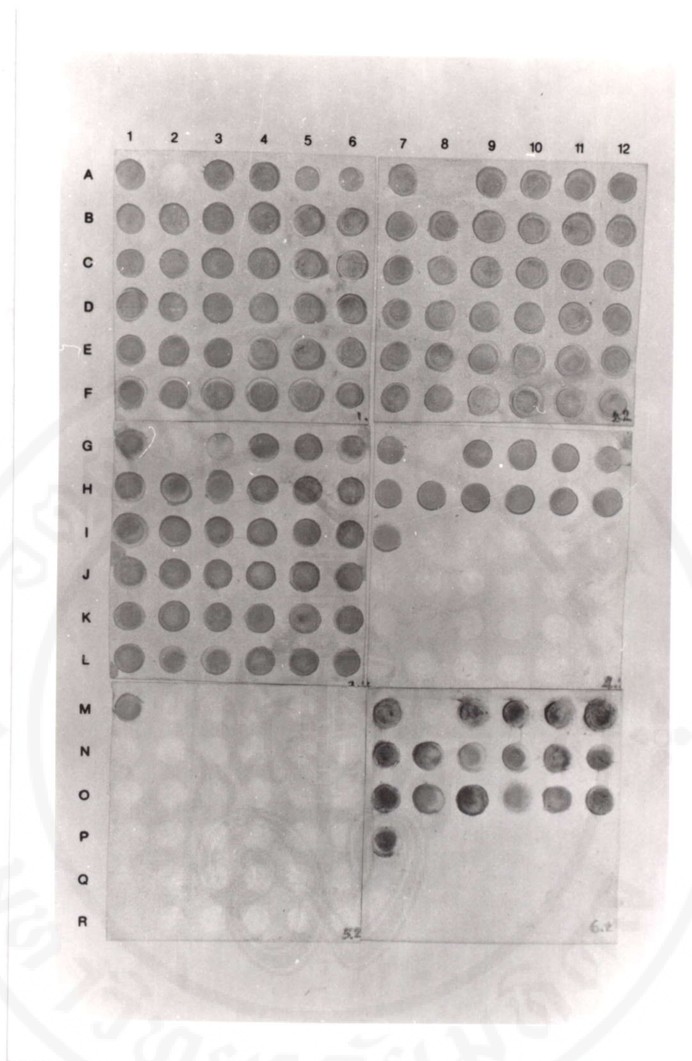

Fig. 18 Dot blot hybridization of *E. coli* and other bacteria with GA549 probe. The types of bacteria and positions on the membrane are the same as described in Fig. 15.

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PCR amplification from intact bacterial cells (around 10^5 to 10^6 cells per reaction) with primers GAL-301 and GAR-432 generated amplified DNA fragments of expected size, a 0.153 kb, from all *E. coli* (lane 2-20, 22-38 in Fig. 19A, B, 21A and lane 2-13 in Fig. 21B) and 11 strains of *Shigella* spp. (lane 14-20, 22-25 in Fig. 21B). Southern blot DNA-DNA hybridization with Dig-labeled DNA probe GA153 showed the positive results of all amplified DNA (Fig. 20 and 22) which indicated that the PCR products were from *uidA* gene. With all other strains of non *E. coli* or non *Shigella* strains, no PCR product was detectable(lane 26-38 in Fig. 21B)

The other pairs of primers, GAL-301 and GAR-806, produced amplified DNA fragments of a 0.527 kb for all tested strains of *E. coli* (Fig. 23A lane 2-21, 23-38, Fig. 23B lane 2-20, 22-38, Fig. 25A lane 2-20, 22-38, and Fig. 25B lane 2-13) and *Shigella* spp. (Fig. 25B lane 14-20, 22-25). Southern blot hybridization analysis with probe GA527 also showed the positive results of all amplified DNA (Fig. 24 and 26). No amplification was observed for other bacterial strains, some of them are shown in Fig. 25B, lane 26-38. The results of DNA amplification of all bacterial strains are compiled in Table 6. Thus, occurrence of an amplification product using indicated primers was limited to the *E. coli* and *Shigella* spp. (Table 6).

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Table 6 PCR amplification directly from intact cells with various bacterial strains.

Microorganisms[a]	No. of strains	PCR[b] amplification with primers	
		GAL-301 and GAR-432	GAL-301 and GAR-806
Escherichia coli	120	+	+
Citrobacter freundii	2	-	-
Citrobacter diversus	1	-	-
Citrobacter spp.	2	-	-
Enterobacter aerugenes	1	-	-
Enterobacter cloacae	4	-	-
Edwardsiella tarda	1	-	-
Klebsiella ozanae	1	-	-
Klebsiella pneumoniae	6	-	-
Morganella morganii	1	-	-
Proteus vulgaris	1	-	-
Pseudomonas putida	1	-	-
Salmonella spp.	43	-	-
Shigella boydii	1	+	+
Shigella flexneri	6	+	+
Shigella sonnei	4	+	+
Bacillus megaterium	1	-	-
Bacillus sphericus	1	-	-
Bacillus subtilis	1	-	-
Bacillus turingiensis	1	-	-
Staphylococcus aureus	2	-	-

[a] About 10^5-10^6 cells/10 μl was used in each PCR reaction.

[b] PCR condition was performed as essentially described in Material and Methods.

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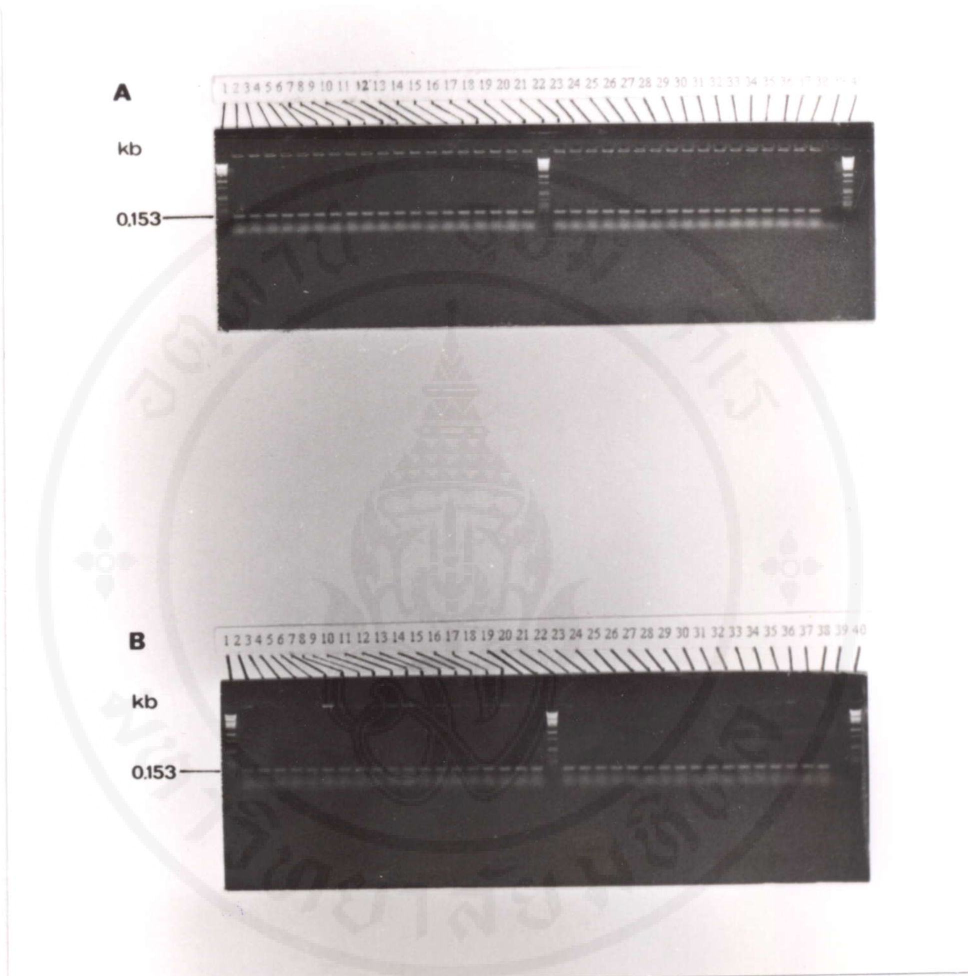

Fig. 19 Ethidium bromide-stained 1.5% agarose gel of a 0.153 kb amplified DNA from 72 strains of *E. coli* No.1-72 listed in Table 5, using primers GAL-301 and GAR-432. Lanes 1, 21 and 40 of both A and B are λ*Pst*I. Lanes 39 of A and B are negative control without DNA template. Lane 2-20, 22-38 of A are *E. coli No*. 1-36 listed in Table 5 and lane 2-20, 22-38 of B are *E. coli* No.37-72 listed in Table 5. The broad bands under the 0.153 kb amplified DNA are primer amplification artifacts caused by excess primers.

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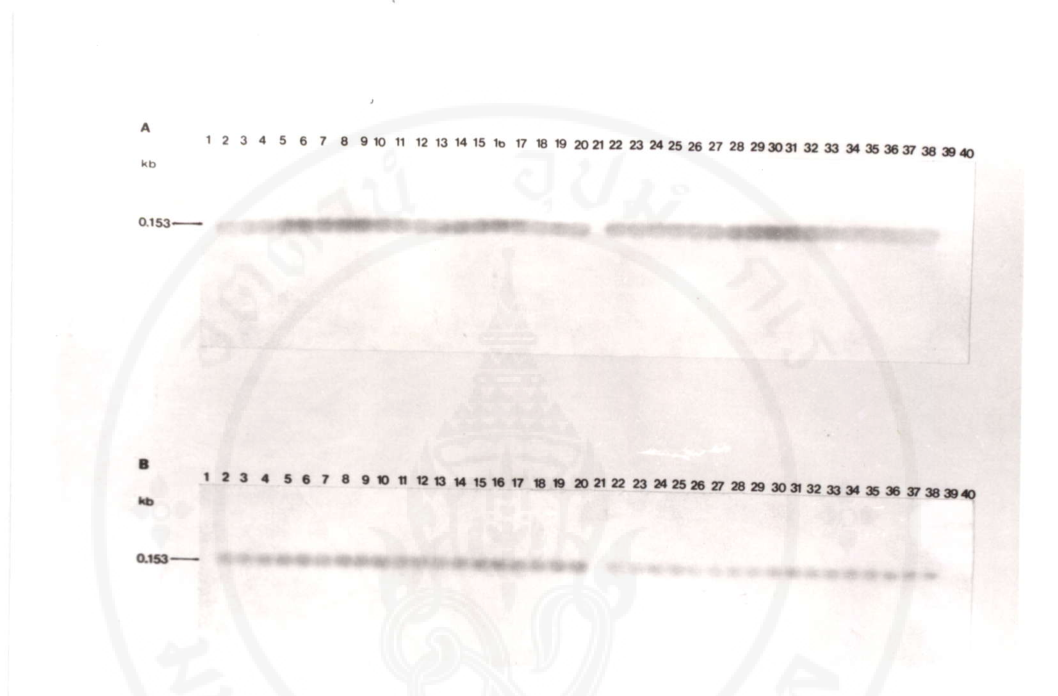

Fig. 20 Southern blot hybridization of the amplified 0.153 kb DNA fragments with Dig-labeled probe GA153. All lanes of both A and B are the same as indicated in Fig. 19.

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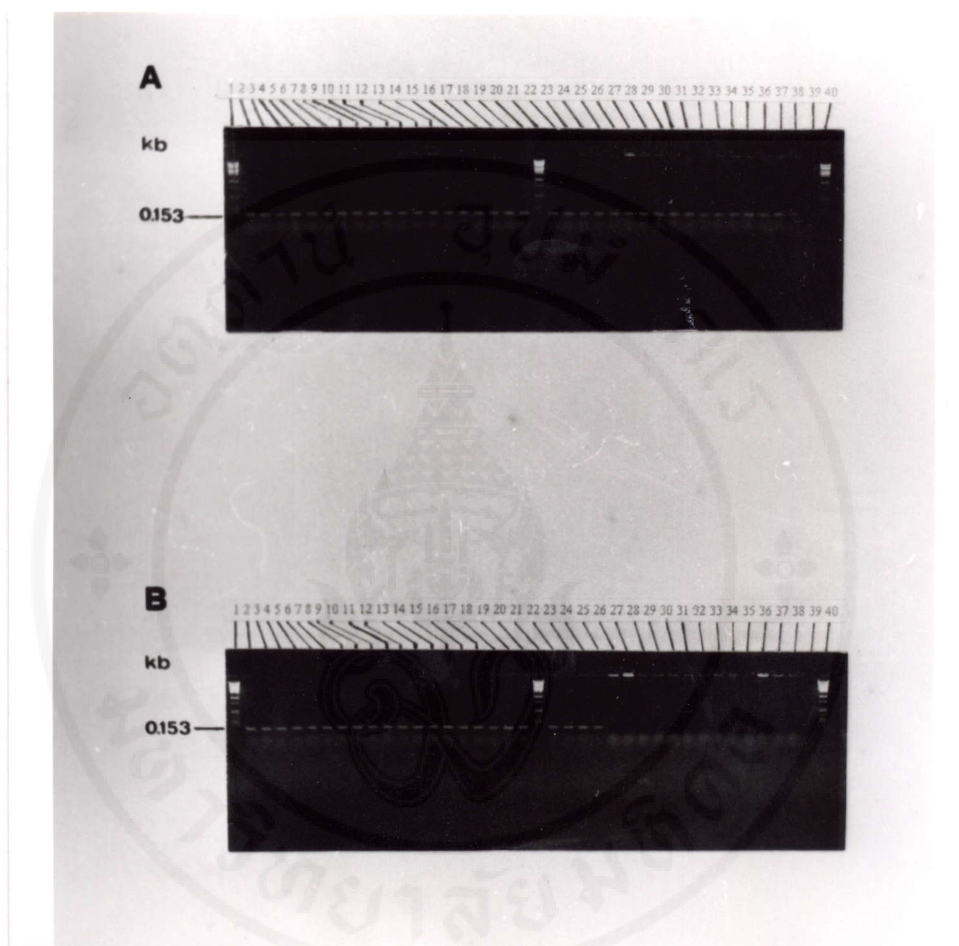

Fig. 21 Ethidium bromide-stained 1.5% agarose gel of a 0.153 kb amplified DNA from 48 strains of *E. coli* No.73-120 and *Shigella* spp. No.185-195 listed in Table 5, using primers GAL-301 and GAR-432. Lanes 1, 21 and 40 of both A and B are λ*Pst*I. Lanes 39 of A and B are negative control without DNA template. Lanes 14-20, 22-25 of B are *Shigella* spp. No.1-36. Lanes 26-38 of B are *Citrobacter freundii*, *C. diversus*, *Enterobacter aerugenes*, *E. cloacae*, *Edwardsiella tarda*, *Klebsiella ozaenae*, *K. pneumoniae*, *Morganella moganii*, *Proteus vulgaris*, *Pseudomonas putida*, *Salmonella* Agona, *Bacillus megaterium* and *Staphylococcus aureus*, respectively. The other lanes are *E. coli*.

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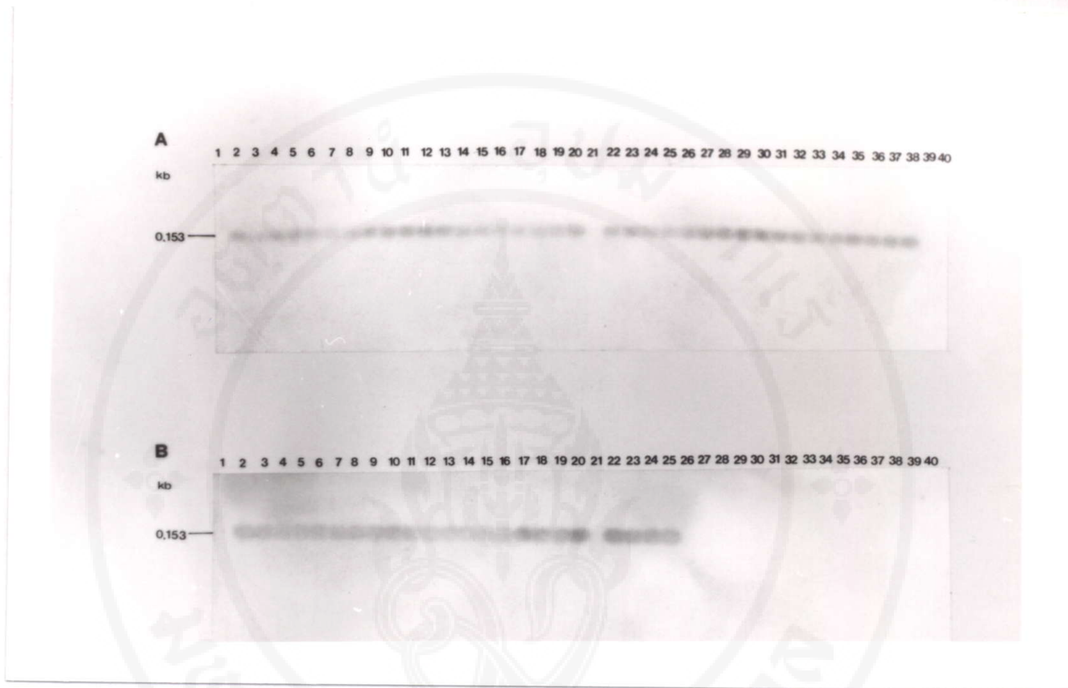

Fig. 22 Southern blot hybridization of the amplified 0.153 kb DNA fragments with Dig-labeled probe GA153. All lanes of both A and B are the same as indicated in Fig. 21.

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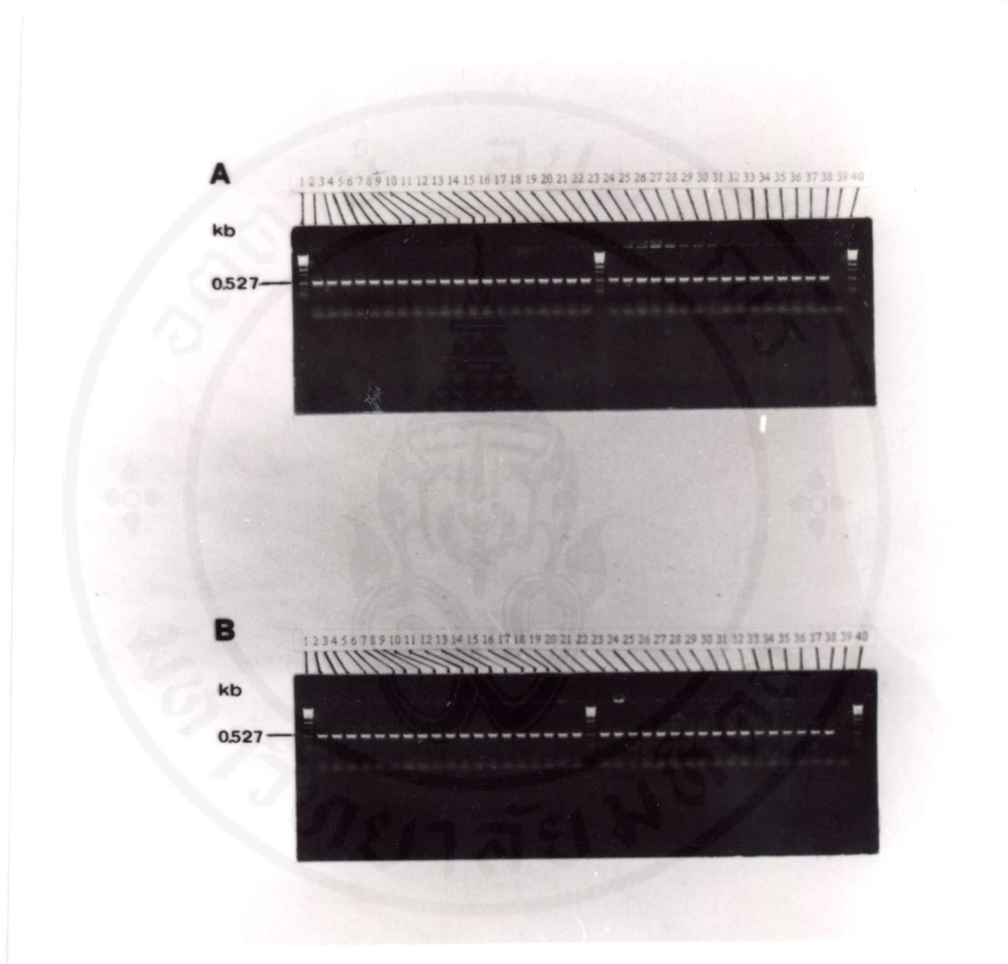

Fig. 23 Ethidium bromide stained 1.5% agarose gel of a 0.527 kb amplified DNA from 72 strains of *E. coli* No.1-72 listed in Table 5, using primers GAL-301 and GAR-806. Lanes 1, 22 and 40 of A, 1, 21, 40 of B are λ*Pst*I. Lanes 39 of A and B are negative control without DNA template. Lane 2-21, 23-38 of A are *E. coli* No.1-36 listed in Table 5 and lane 2-20, 22-38 of B are *E. coli* No. 37-72 listed in Table 5. The broad bands under the 0.527 kb amplified DNA are primer amplification artifacts caused by excess primers.

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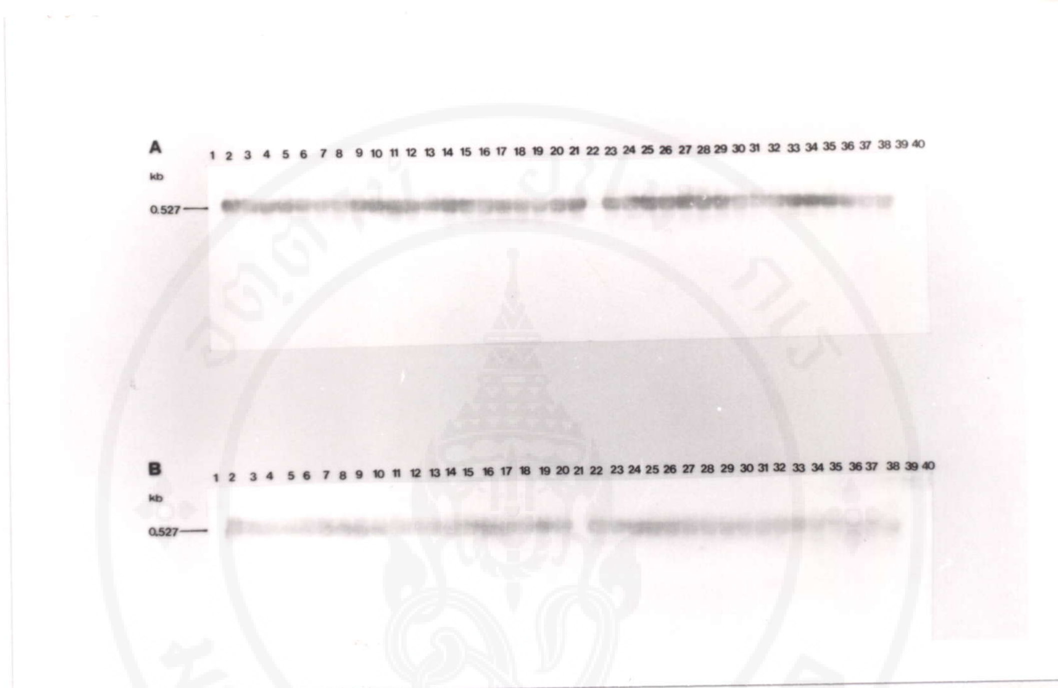

Fig. 24 Southern blot hybridization of the amplified 0.527 kb DNA fragments with Dig-labeled probe GA527. All lanes of both A and B are the same as indicated in Fig. 23.

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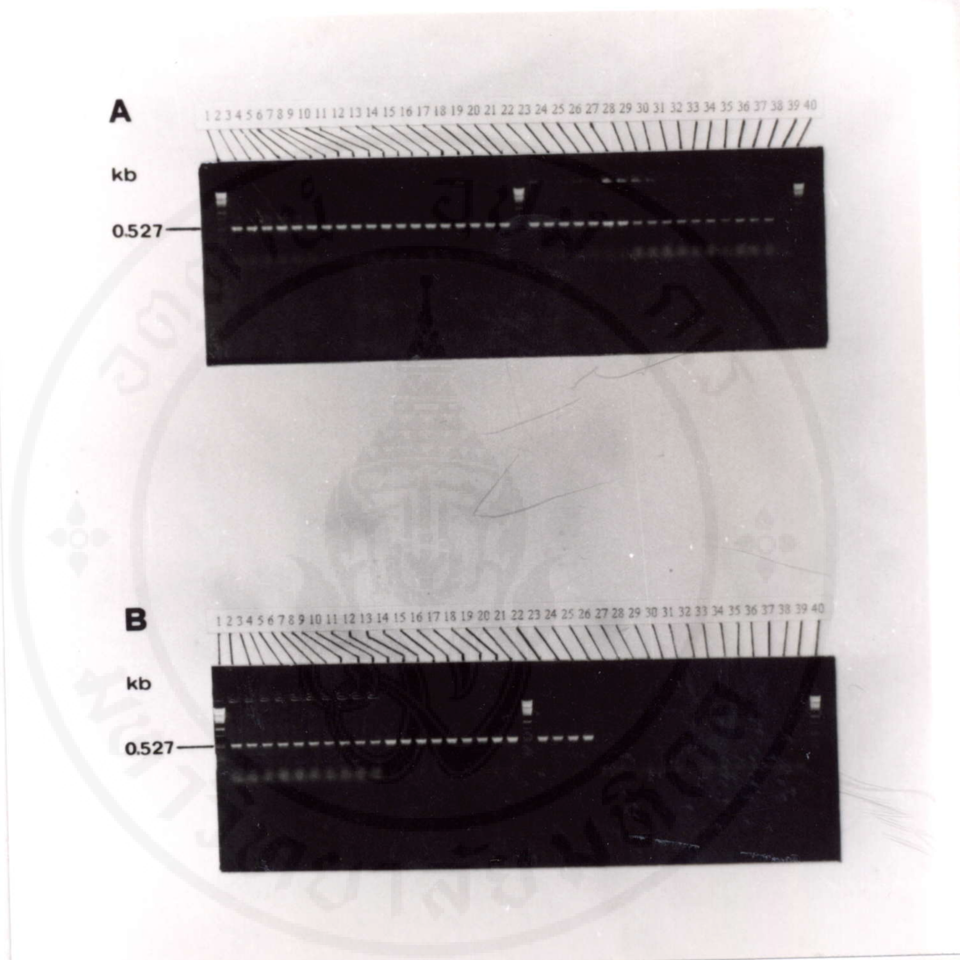

Fig. 25 Ethidium bromide stained 1.5% agarose gel of a 0.527 kb amplified DNA from 48 strains of *E. coli* No.73-120 and *Shigella* spp. No.185-195 listed in Table 5, using primers GAL-301 and GAR-806. Lanes 1, 21 and 40 of both A and B are λ*Pst*I. Lanes 39 of A and B are negative control without DNA template. Lanes 14-20, 22-25 of B are *Shigella* spp. No.1-36. Lane 26-38 of B are *Citrobacter freundii*, *C. diversus*, *Enterobacter aerugenes*, *E. cloacae*, *Edwardsiella tarda*, *Klebsiella ozaenae*, *K. pneumoniae*, *Morganella moganii*, *Proteus vulgaris*, *Pseudomonas putida*, *Salmonella* Agona, *Bacillus megaterium* and *Staphylococcus aureus*, respectively. The other lanes in both A and B are *E. coli*.

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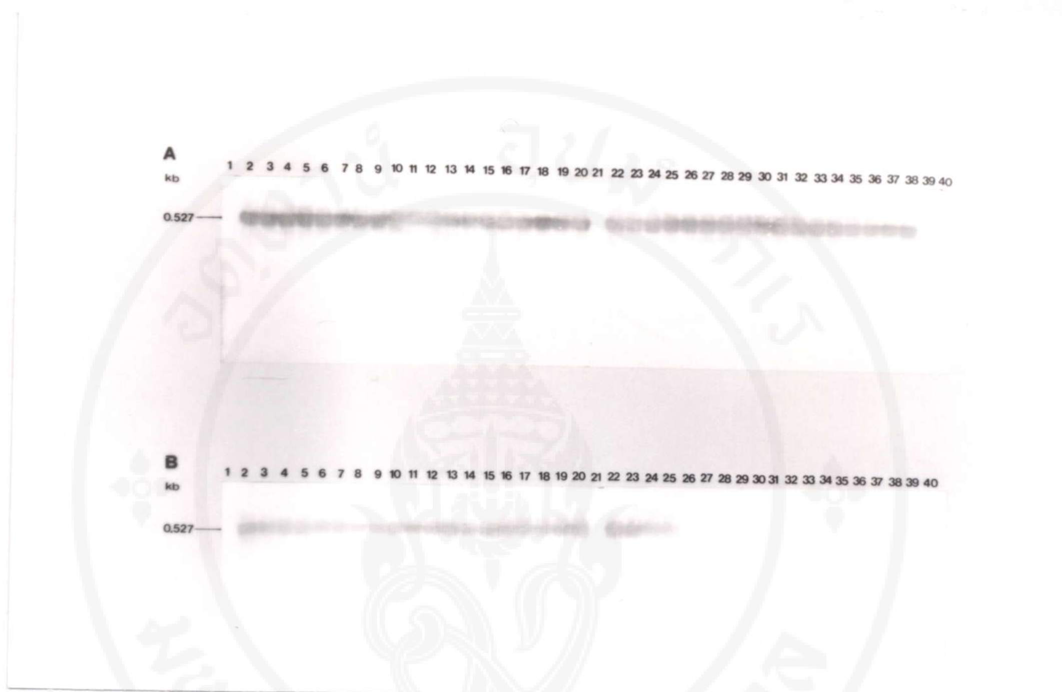

Fig. 26 Southern blot hybridization of the amplified 0.527 kb DNA fragments with Dig-labeled probe GA527. All lanes of both A and B are the same as indicated in Fig. 25.

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5. Detection of *E. coli* in water samples.

Since DNA fragment from *uidA* gene of all 120 strains of *E. coli* could be amplified and produced DNA fragments of a 0.153 and a 0.527 kb in size by using 2 pairs of primers as stated above. Therefore, the technique might be used to detect *E. coli* present in water or food samples as well.

Drinking water samples from 10 brands available in Faculty of Science, Mahidol University and Ramathibodi Hospital were examined by viable plate count, membrane filter culture technique on Fluorocult ECD agar and PCR amplification. One milliliter of undiluted and their ten-fold serial diluted samples was used for pour plating using synthetic Fluorocult plate count agar. After total bacterial cell count, these plate were observed under UV light (366 nm) to search for fluorescent colonies which were expected to be *E. coli*.

For membrane filter culture technique on Fluorocult ECD agar, a 100 ml of water samples no. 1 and 2 were filtered through HA membrane filters easily (47 mm in diameter and 0.45 μm-pore-size). But 100 ml of samples number 3-10 were very difficult to be filtered for one time through such membrane, because of their high turbidity. Therefore, the following procedure was performed in a similar fashion for each of the samples number 3-10. Each sample was divided into five portions of 20 ml each. Each portion was filtered through separated HA membranes. Subsequently these filtered membranes were placed over (colony side up) Fluorocult ECD agar and examined for the appearance of fluorescent colonies under long wave UV light after incubation for 24-48 hrs. Autoclaved distilled water was used as negative control.

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The number of bacterial cells and the present of fluorescent colonies on Fluorocult plate count agar and Fluorocult ECD agar were shown in Table 7. Five water samples contained bacterial count ranged from 1.6-7.3 x 10^3 cells/ml and 3 samples contained even higher bacterial count of 1.8-8.9 x 10^4 cells/ml. The bacteria presented in water samples were not *E. coli* since the tested by either plate count in combination with fluorescence on media containing MUG and indole observation were all negative. However, the types of those bacteria were not identified and one example of those colonies on Fluorocult ECD agar was shown in Fig. 27. The bacterial colonies contaminated in water sample number 10 were not fluorescent under UV light (Fig. 27). In order to checked technique of filter culture, the positive control was performed by using *E. coli* K12. Fig. 28 show positive control which was autoclaved distilled water artificially contaminated with *E. coli* K12 (73 cells/100 ml of water). As shown in the figure, *E. coli* give fluorescent colonies on Fluorocult ECD agar under UV light. The presence of *E. coli* was further confirmed by detection of indole formation by reacting with KOVACS' indole reagent and red color reagent was observed which demonstrated that those colonies were *E. coli* as shown in Fig. 29.

To confirm the results of bacterial detection by plate count with both filter culture and pour plate, PCR method was also used to detect *E. coli* in these water samples. Each water sample was filtered through FHLP membrane (13 mm diameter and 0.5 µm-pore-size) using the same water volumes as used in filtration through HA membrane. These filtered membranes with cells-coated on, were heated in boiling water for 5 min to lyse cells and then were subjected directly to PCR amplification by using the two pairs of primers, GAL-301 with GAR-432 and GAL-301 with

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Table 7 Detection of *E.coli* in drinking water samples.

Water samples	Plate count/ml	No.of fluorescence colony on		PCR result
		Fluorocult plate count agar	Fluorocult ECD agar	
1.Singha	0	0	0	-
2.Polaris	0	0	0	-
3.Toto	$8.9x10^4$	0	0	-
4.Pimpilalai	$4.8x10^4$	0	0	-
5.Bandit	$3.9x10^3$	0	0	-
6.Jackpot	$1.8x10^4$	0	0	-
7.Ranong	$1.6x10^3$	0	0	-
8.Spring	$4.3x10^3$	0	0	-
9.Waterle	$5.0x10^3$	0	0	-
10.Faculty of science's water tank	$7.2x10^3$	0	0	-

- negative result.

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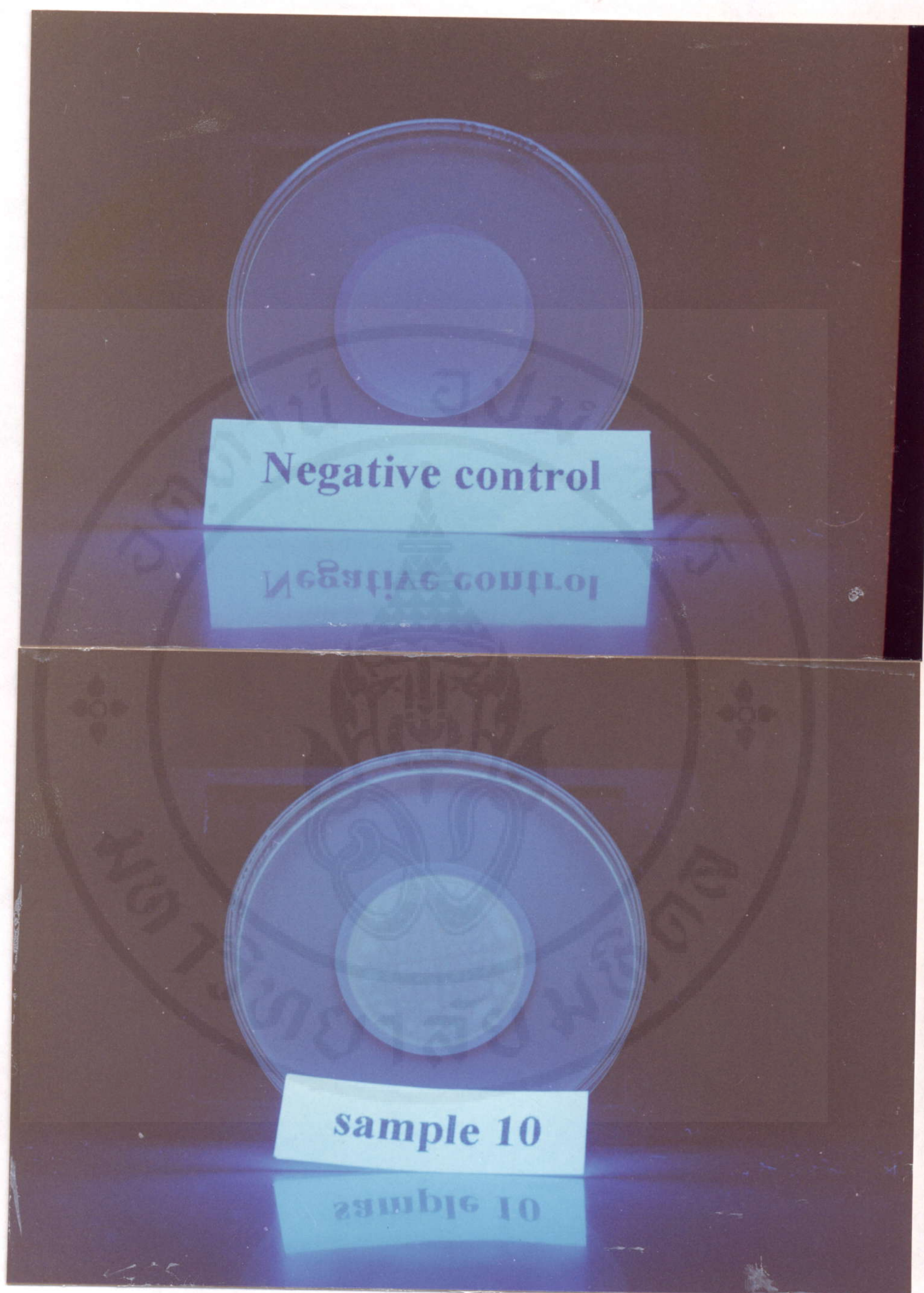

Fig. 27 Photograph of HA membranes placed over Fluorocult ECD agar plates showing the negative control (upper) and colonies from water sample No.10 (lower) which displayed non-fluorescence appearance.

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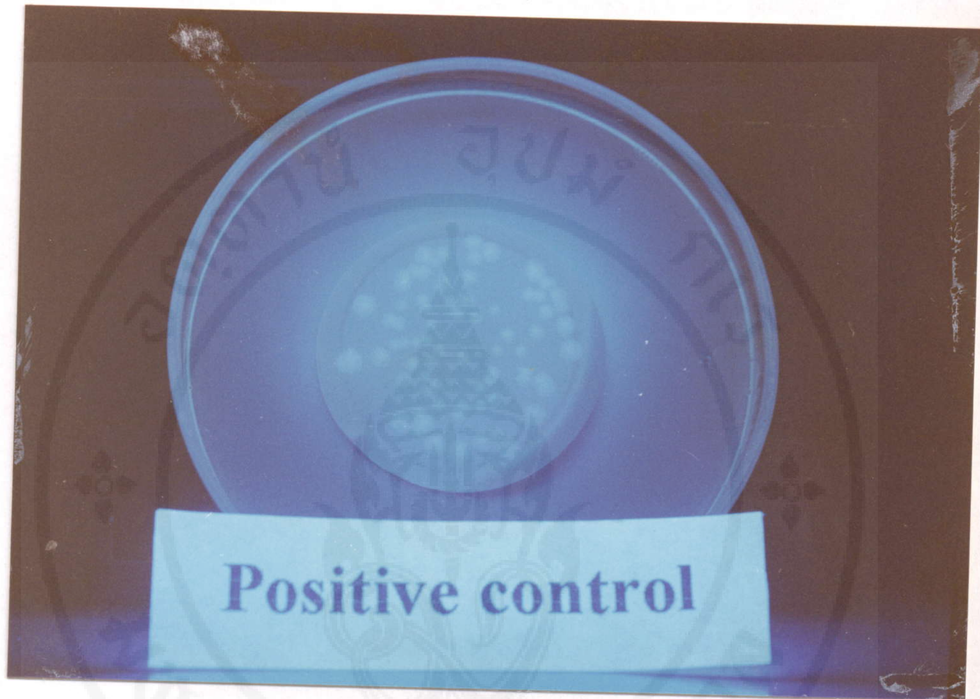

Fig. 28 Photograph of a HA membrane placed over Fluorocult ECD agar plate showing the fluorescence colonies of *E. coli* K12 under long-wave UV light.

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Fig. 29 Photograph of a HA membrane placed over Fluorocult ECD agar plate showing the indole positive with KOVACS' reagent dropped over *E. coli* colonies. The reagent turns red upon reacted with indole produced by *E. coli*.

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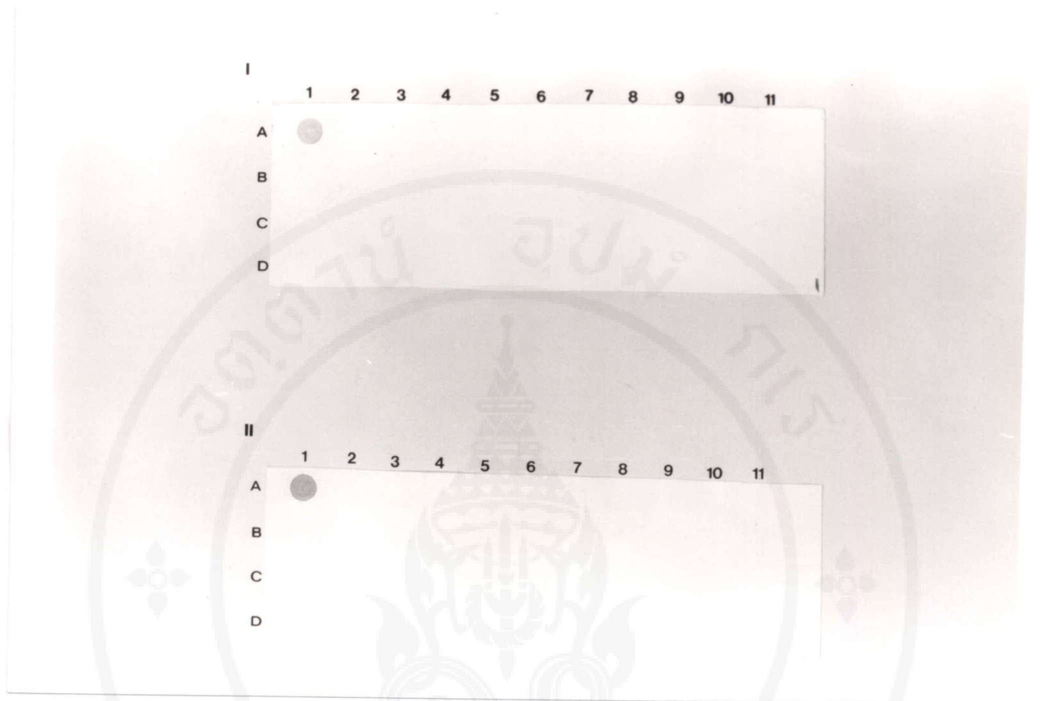

Fig. 30 Dot blot hybridization of PCR products from ten water samples using probe GA153 (I) and GA527 (II). PCR amplification was performed by using primers GAL-301 with GAR-432(I) and primers GAL-301 with GAR-806 (II). No hybridization signal was detected with PCR products from all water samples. A1 is *E. coli* K12, A2 is negative control, A3 is sample No.1, A4 is sample No.2, A5-A9 are sample No.3, A10-A11 and B1-B3 are sample No.4, B4-B8 are sample No.5, B9-B11 and C1-C2 are sample No.6, C3-C7 are sample No.7, C8-C11 and D1 are sample No.8, D2-D6 are sample No.9 and D7-D11 are sample No.10.

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GAR-806. After amplification 20 μl of PCR products were electrophoresed and about 120 μl were dotted on nylon membrane and hybridized with probe GA153 and GA527. No PCR products were observed (Table 7) by both gel electrophoresis (not shown) and dot blot hybridization (Fig. 30). Thus *E. coli* was not detected in the 10 water samples by either pour plating, filtration culture and PCR techniques.

6. Detection of *E. coli* in food sample.

To test the techniques of culture method, hybridization and PCR for detection of *E. coli* present in food samples, frozen boiled octopus was used for testing. Twenty five gram of frozen boiled octopus was prepared by homogenization in 225 ml of LB-broth. The total aerobic microbial count of this sample on Fluorocult plate count agar revealed the presence of bacterial cells of 8.8 x 10^3 CFU/g. Therefore, it is indicated that the boiled octopus was still contaminated with some bacteria. Multiple-tube fermentation technique with Fluorocult Lauryl Sulfate Broth which was conventional method for detection of *E. coli* was used. A 3-tube set for each dilution of octopus samples was used for determination of most probable number (MPN) by inoculation of samples with serial dilution of 1:10, 1:100 and 1:1,000 dilution of food homogenate. All dilutions of food homogenate tested did not fluoresce under longwave UV light (366 nm) after growing at 37^0C for 48 hrs and the 1;10 dilution of food homogenate was shown in Fig. 31-B. Whereas positive control with artificially contaminated *E. coli* K12 showed fluorescence under UV light (Fig. 31-A). To confirm the present of *E. coli* in culture broth, indole test was performed and the red color in the KOVACS'

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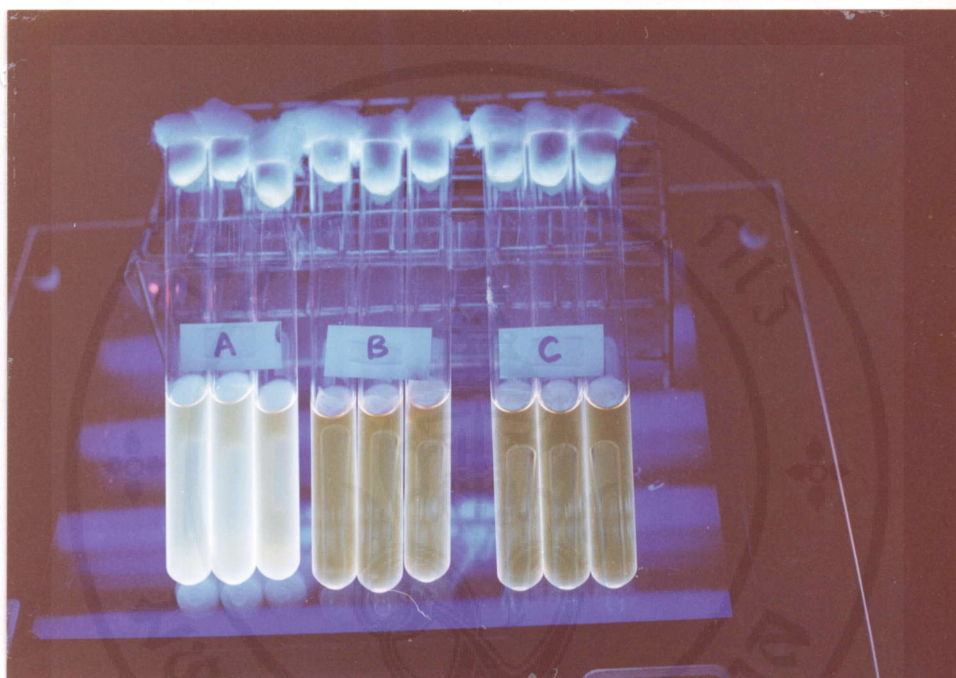

Fig. 31 Photograph of three sets of Fluorocult Lauryl Sulfate Broth tubes inoculated with triplicate set of food sample contaminated with *E. coli* (A), 1:10 dilution of food sample (B) and negative control without food sample (C). The tubes were seen under UV light after incubation at 37°C for 48 hrs. Fluorescence is observed only in A set which contained *E. coli* K12.

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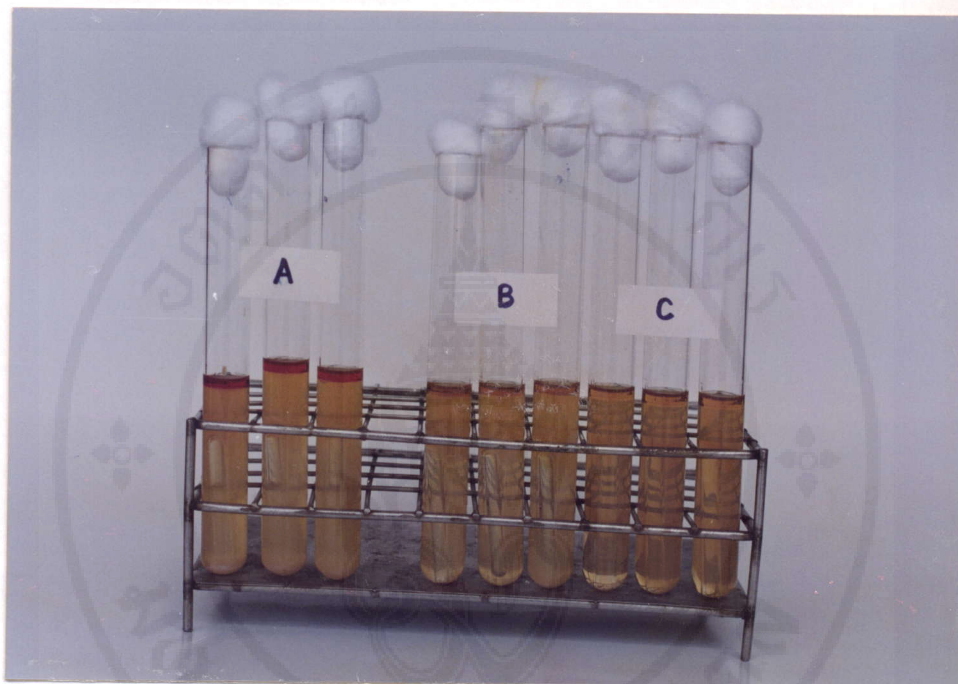

Fig. 32 Photograph of three sets of Fluorocult Lauryl Sulfate Broth tubes inoculated with triplicate set of food sample contaminated with *E. coli* (A), 1:10 dilution of food sample (B) and negative control without food sample (C) after incubation at 37°C for 48 hrs and addition of KOVAC's reagent. The red color layer was observed only in A set which indicated the indole formation from *E. coli* present in the culture broth.

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indole reagent layer was detected only in sample contained *E. coli* as shown in Fig. 32-A. No red color reagent was detected in food sample alone (Fig. 32-B). Thus *E. coli* was not detected in this food sample by using the conventional cultural method. The bacterial count of 8.8 x 10^3 CFU/g present in food sample were not *E. coli* as judged by no fluorescence under UV light (Fig. 31-B) and no indole reaction (Fig 32-B).

By using PCR technique to detect *E. coli* in food sample, two procedures were comparatively performed by using the same food homogenate prepared above (25 g in 225 ml LB broth). The first procedure was filtration one milliliter of the food sample through FHLP membrane and after cells lysis, the membrane was subjected to PCR assay. The other procedure was performed by direct PCR amplification with 10 µl of boiled food homogenate. Both two sets of primers, GAL-301 with GAR-432 and GAL-301 with GAR-806, were used in these experiments. Two types of food samples tested by these two PCR methods were the original food sample and *E. coli* artificially contaminated food sample. Both types of samples were preenriched by incubating separately at 37°C for 0, 3 and 6 hrs in rotary shaker. At each indicated time, the samples were assayed for the presence of *E. coli* by both culture and PCR techniques. It is found that no *E. coli* was detected in original food samples incubated at 0, 3 and 6 hrs by either culturing on Fluorocult ECD agar or PCR amplification of these food samples as detected by gel electrophoresis (data not shown). However, to confirm the result, dot blot hybridization of PCR products from each reaction was performed. Fig. 33 showed that dot blot hybridization of PCR products of the original food sample incubated at 0, 3 and 6 hrs using either PCR procedures were all negative. Positive

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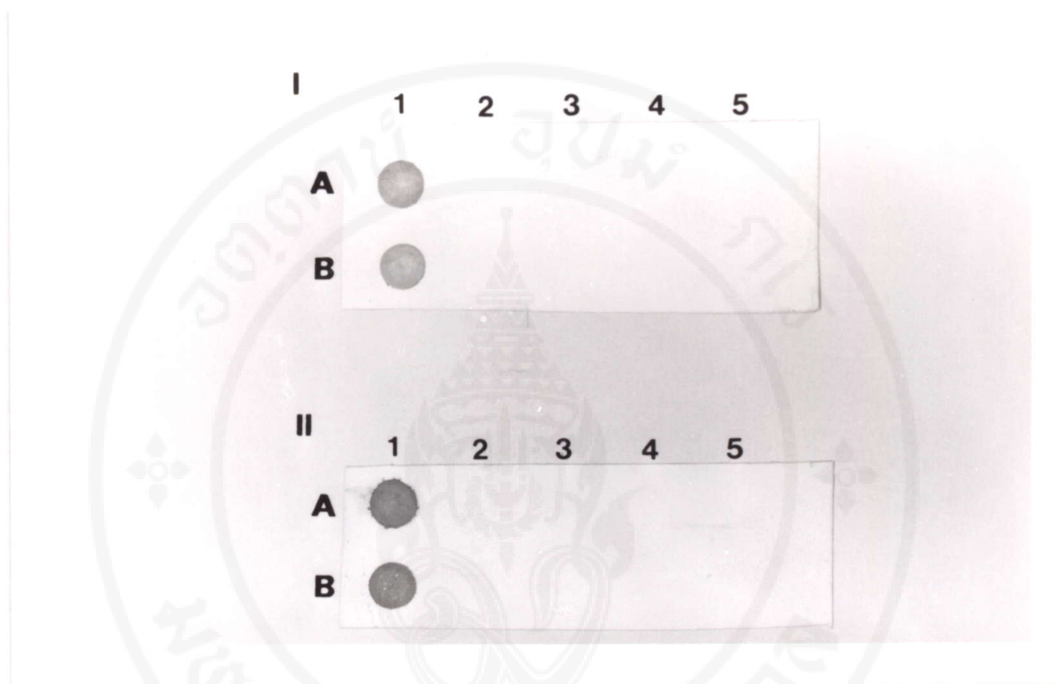

Fig. 33 Dot blot hybridization of PCR products from original food homogenate using primers GAL-301 with GAR-432 (I) and primers GAL-301 with GAR-806 (II). Row A, the food samples were processed by sample filtration followed by PCR. Row B, PCR amplification directly from boiled homogenate of food sample. A1 and B1 are *E. coli* K12 (positive control). A5 and B5 are negative control (no food sample). A2-A4 and B2-B4 are original food homogenate incubated at 37°C for 0, 3 and 6 hrs, respectively before PCR performance.

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signals occurred only from PCR amplification of samples contained *E. coli* cells (Fig. 33 Row A1 and B1). This result together with culture method indicated that the original food homogenate did not contain *E. coli* cells.

The number of *E. coli* cells from artificially contaminated food samples and the result of PCR reactions with preenrichment step were shown in Table 8. The longer the incubation time, the larger the amount of amplified DNA. For example, PCR products obtained from sample incubated for 6 hrs (Fig. 34 lane 7) was more than that PCR products obtained from sample incubated for 1 or 3 hrs (Fig. 34 lane 5, 6). The similar results were obtained from both pairs of primers. Fig. 34 and 35 showed gel electrophoresis of PCR products of the artificially contaminated food samples by using both PCR procedures. The intensity of PCR products from both PCR methods are not different but will increase upon longer incubation (Fig. 34 and 35 lanes 4-7, lanes 8-11). The dot blot hybridization of those PCR products from each reaction was shown in Fig. 36. All samples with contaminated *E. coli* showed positive signals as *E. coli* positive control. Hence, the amplified products of DNA fragments from food samples were from *uidA* gene of *E. coli*.

The other experiment with the objective to study the possibility of PCR technique for detection of free DNA released from nonviable cells contaminated in food sample was also performed. Three aliquots of one milliliter of *E. coli* free food samples as determined by culture method, hybridization and PCR techniques were mixed with 1 ng of *E. coli* K12 chromosomal DNA. This amount of DNA is equivalent to 10^6 bacterial cells (Bej et al, 1991c) and then the mixture was incubated at 37°C for 0, 30 and 60 min. These samples were centrifuged and the pellets were suspended in 100 μl

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of autoclaved deionized distilled water and mixed with 50 μl of PCR reaction mixture then subjected to PCR amplification. The other set of samples was also analyzed by PCR technique but filtration technique with FHLP membrane was performed instead of sample centrifugation. PCR products were detected by gel electrophoresis (Fig. 37) and dot blot hybridization (Fig. 38). Samples filtration and centrifugation give no different results. PCR technique can amplify 1 ng of *E. coli* chromosomal DNA contaminated in food sample even after incubation of the sample at 37°C for 0, 30 and 60 min.

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Table 8 *E.coli* counts and PCR products obtained from artificially contaminated food samples.

Time (hr)[a]	*E.coli* CFU/ml	PCR product with primers[b]	
		GAL-301 and GAR-432	GAL-301 and GAR-806
0	9.0×10^1	+	+
1	4.7×10^2	++	++
3	8.4×10^4	+++	+++
6	9.5×10^6	++++	++++

[a] The food samples mixed with *E. coli* was incubated at 37°C as indicated time prior to PCR analysis.

[b] Similar results were found for both methods of PCR amplification as described in text.

+, weak amplification; ++, moderate amplification; +++, strong amplification ; ++++, very strong amplification.

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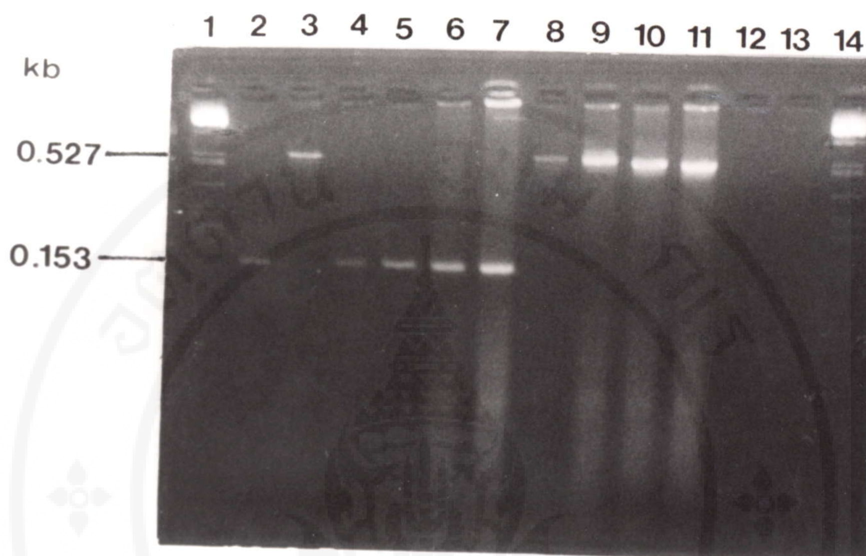

Fig. 34 Ethidium bromide-stained 4% Nusieve (3:1) agarose gel of PCR products from artificially contaminated food sample as performed by filtration 1 ml of sample through FHLP membrane. Lanes 1 and 14 are λ*Pst*I. Lanes 2 and 3 are positive control of a 0.153 and a 0.527 kb PCR products, respectively. Lanes 12 and 13 are negative control (no food samples). Lanes 4-7 are PCR products of reactions with primers GAL-301 and GAR-432 from the samples incubated for 0, 1, 3 and 6 hrs, respectively. Lanes 8-11 are PCR products of reactions with primers GAL-301 and GAR-806 from the samples incubated for 0, 1, 3 and 6 hrs, respectively. Only 20 μl out of 150 μl of total PCR product were loaded in each lane.

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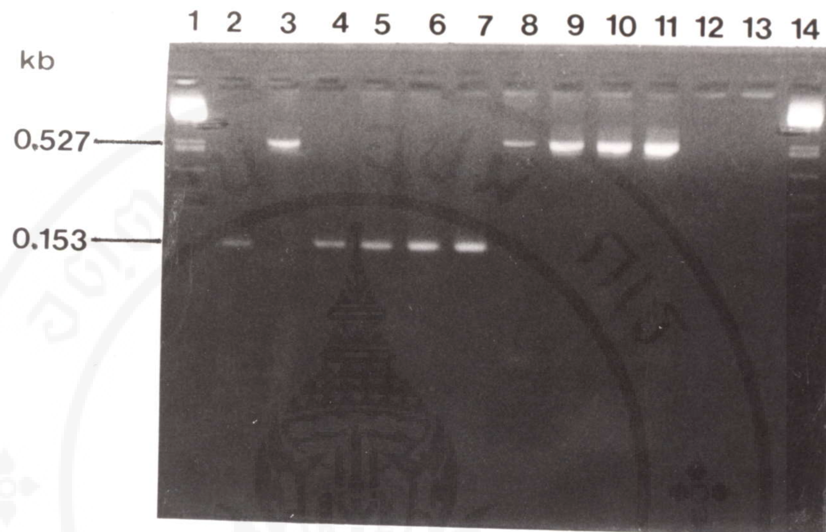

Fig. 35 Ethidium bromide-stained 4% Nusieve (3:1) agarose gel of PCR products from artificially contaminated food sample as performed by direct PCR amplification of a 10 μl from 1 ml boiled food homogenate mixed with *E. coli*. Lanes 1 and 14 are λ*Pst*I. Lanes 2 and 3 are positive control of a 0.153 and a 0.527 kb PCR products, respectively. Lanes 12 and 13 are negative control (no food samples). Lanes 4-7 are PCR products of reactions with primers GAL-301 and GAR-432 from the samples incubated for 0, 1, 3 and 6 hrs, respectively. Lanes 8-11 are PCR products of reactions with primers GAL-301 and GAR-806 from the samples incubated for 0, 1, 3 and 6 hrs, respectively. Only 20 μl out of 100 μl of PCR products were loaded in each lane.

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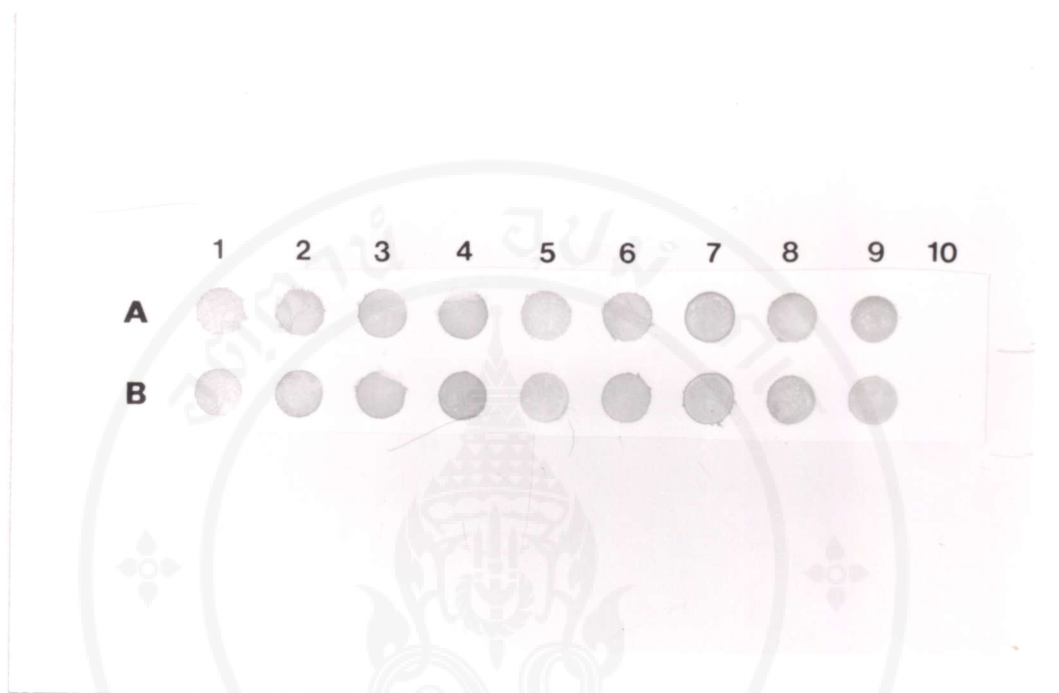

Fig. 36 Dot blot hybridization of PCR products from artificially contaminated food sample as performed by sample filtration through FHLP membrane (A) or direct PCR using a 10 μl of boiled food homogenate (B). The Dig-labeled-GA153 was used as probe. A9 and B9 are positive control (PCR from intact cells). A10 and B10 are negative control (no food samples). A1-A4 and B1-B4 are PCR products of reactions with primers GAL-301 and GAR-432 incubated for 0, 1, 3 and 6 hrs, respectively. A5-A8 and B5-B8 are PCR products of reactions with primers GAL-301 and GAR-806 incubated at 37°C for 0, 1, 3 and 6 hrs, respectively. Each dot on Row A and B contained 120 μl and 70 μl of PCR products, respectively.

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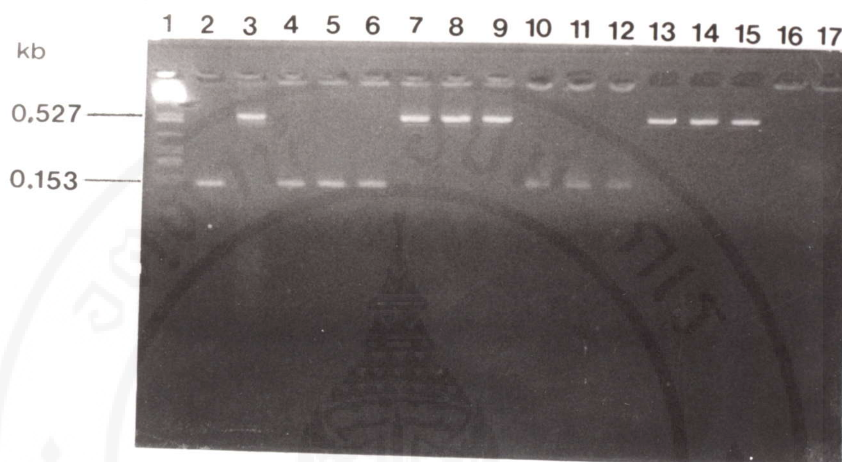

Fig. 37 Ethidium bromide-stained 4% Nusieve (3:1) agarose gel of PCR products from a 1 ml *E. coli* free food sample mixed with 1 ng of *E. coli* chromosomal DNA. The PCR reaction was performed from samples prepared by centrifugation of whole sample (lanes 4-9) or whole sample filtration through FHLP membrane (lanes 10-15). Lanes 1 is λ*Pst*I. Lanes 2 and 3 are positive control of a 0.153 and a 0.527 kb PCR products, respectively. Lanes 4-6 and 10-12 are PCR products of reactions with primers GAL-301 and GAR-432 in which the samples were incubated for 0, 30, 60 min, respectively. Lanes 7-9 and 13-15 are PCR products of reactions with primers GAL-301 and GAR-806 in which the samples incubated at 37°C for 0, 30, 60 min, respectively. Lanes 16 and 17 are negative control. Only 20 μl out of 150 μl of total PCR products were applied in each lane.

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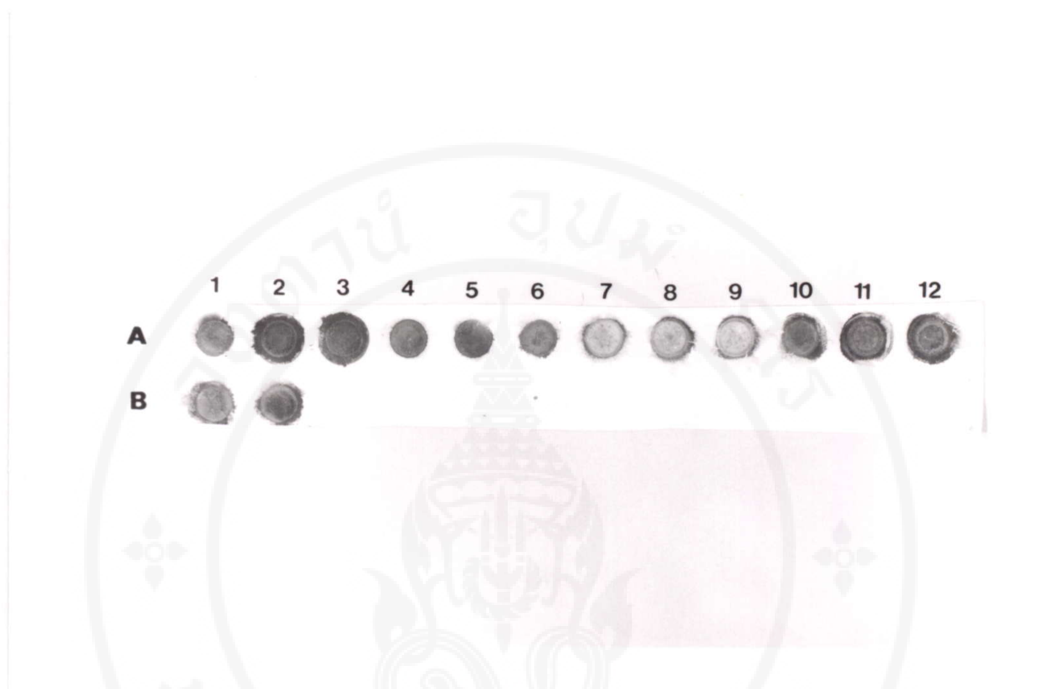

Fig. 38 Dot blot hybridization of PCR products from *E. coli* free food sample mixed with 1 ng of 1 ml *E. coli* chromosomal DNA. The PCR reaction was performed from samples prepared by either whole sample centrifugation (A1-A6) or whole sample filtration through FHLP membrane (A7-A12). The hybridized probe was GA153. A1-A3 and A7-A9 are PCR products of reactions with primers GAL-301 and GAR-432 in which the samples were incubated for 0, 30, 60 min, respectively. A4-A6 and A10-A12 are PCR products of reactions with primers GAL-301 and GAR-806 in which the samples incubated for 0, 30, 60 min, respectively. B1 and B2 are positive control (PCR products from intact *E. coli* cells). B3 and B4 are negative control and B5-B12 are blank. Each dot contained 120 µl of PCR products.

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7. Sensitivity of *E. coli* detection.

Experiments to evaluate sensitivity of *E. coli* detection with DNA hybridizaton were performed to determine the minimum number of *E. coli* cells which could be detected by both procedures, dot blot hybridization and PCR amplification.

Sensitivity test for *E. coli* detection determined by dot blot hybridization with digoxigenin labeled DNA probes from *uidA* gene, probes GA175 and GA549, were performed on nylon membranes dotted with 100 μl each of ten-fold serial dilutions of overnight *E. coli* K12 culture diluted in 0.85% normal saline and these membranes were denatured and neutralized before hybridization. *Pseudomonas putida* 12633 was used as negative control. Fig. 39 shows the hybridization results, The two probes, GA175 (Fig. 39A) and GA549 (Fig. 39B), could detect up to 10^5 *E. coli* per dot as determined by the parallel plate count of colony forming units. The sensitivity of these two probes for *E. coli* detection in water was determined by the same method but 0.85% normal saline which used as diluent was replaced with autoclaved distilled water. The hybridization results show that at least 10^5 *E. coli* cells per dot were required for a positive result with the two probes, GA175 (Fig. 40A) and GA549 (Fig. 40B). This method was also used to determine the sensitivity for detection *E. coli* in food sample. The artificially contaminated food samples which were the mixture between 4.5 ml of *E. coli* free food samples and 0.5 ml of ten-fold serial dilutions of overnight *E. coli* K12 culture were dotted on nylon membrane and hybridized with these two probes. The hybridization results were shown in Fig. 41. The similar results were achieved by using these two probes with a detection limit of

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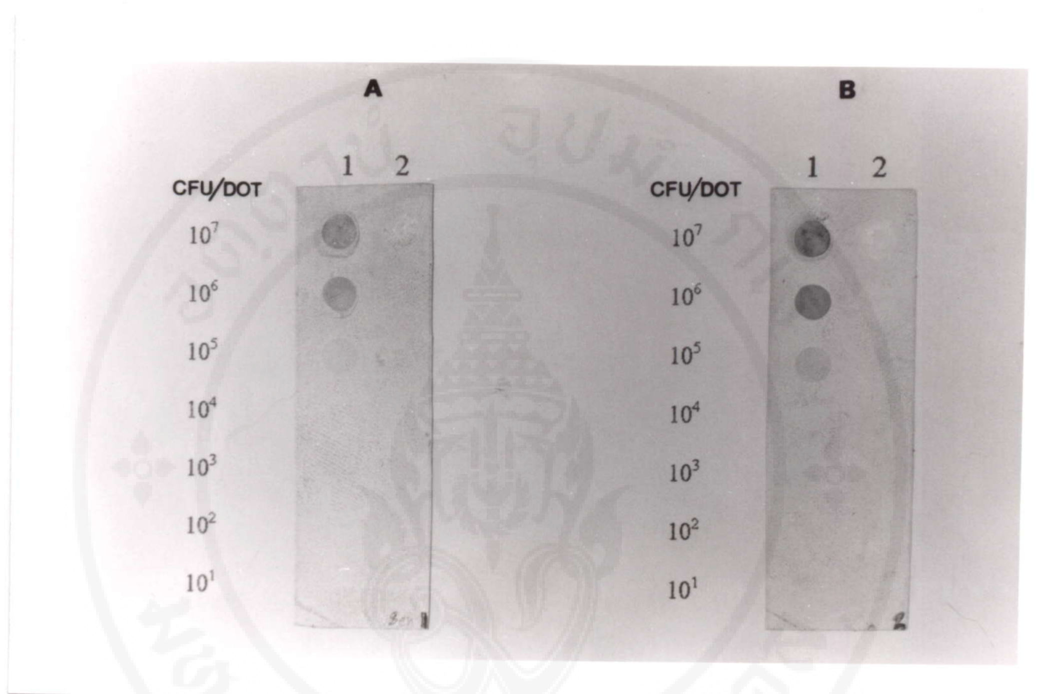

Fig. 39 Dot blot hybridization of serial dilution of *E. coli* K12 overnight culture in 0.85% normal saline using probe GA175 (A) and probe GA549 (B). Row 1 in both panels, A and B, are *E. coli* and row 2 are *Pseudomonas putida* 12633.

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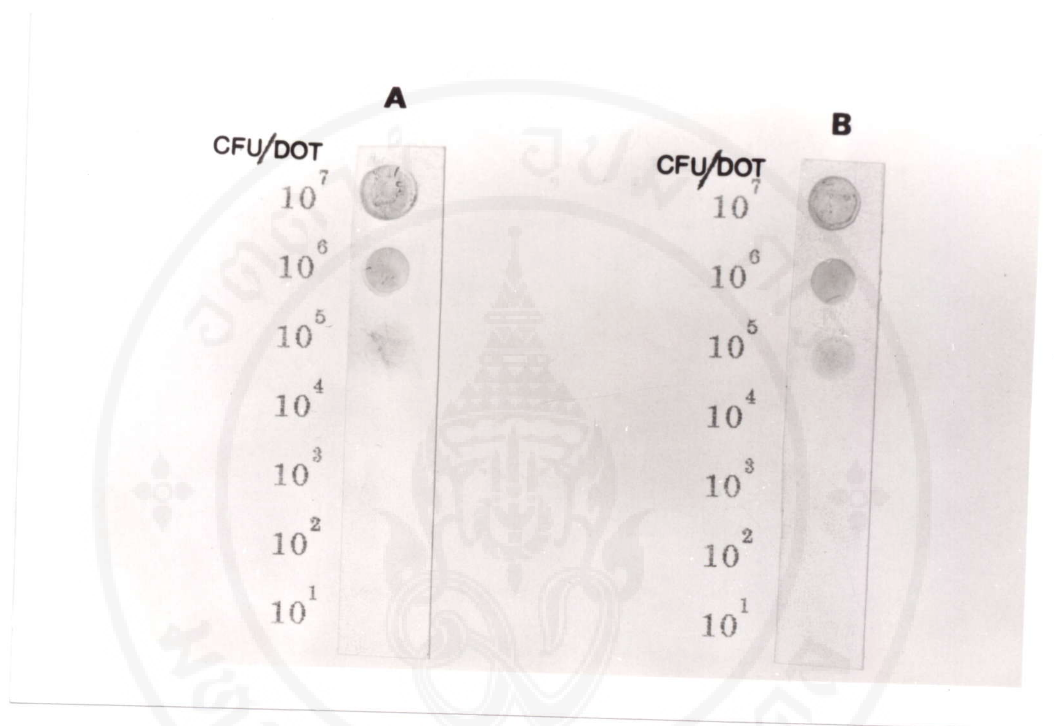

Fig. 40 Dot blot hybridization of serial dilution of *E. coli* K12 overnigh culture in autoclaved distilled water using probe GA175 (A) and probe GA549 (B).

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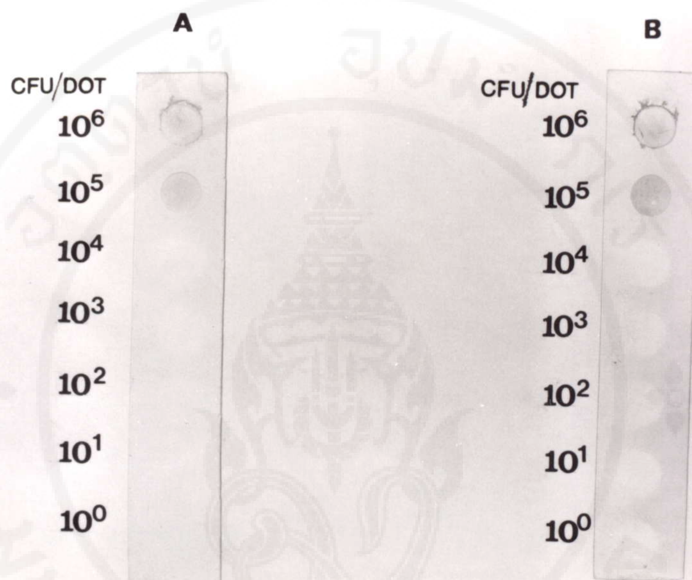

Fig. 41 Dot blot hybridization of serial dilution of overnight *E. coli* culture added to octopus food sample using probe GA175 (A) and probe GA549 (B).

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10^5 cells per dot. Thus the maximum sensitivity of the two sizes of digoxigenin labeled DNA probes from *uidA* gene, GA175 and GA549, as determined by dot blot hybridization technique is at least 10^5 *E. coli* cells per dot.

For determination of sensitivity of PCR technique in *E. coli* detection, one milliliter of each ten-fold serial dilution of *E. coli* K12 in 0.85% normal saline was filtered through a 13-mm-diameter Fluoropore membrane (FHLP; 0.5 µm pore size). The membranes with cells-coated on were suspended in 100 µl of autoclaved deionized distilled water and heated in boiling water to lyse cells and then mixed with 50 µl of PCR reaction mixture before PCR amplification. PCR amplification was performed with primers GAL-301 and GAR-432 which provided a 0.153 kb product and with primers GAL-301 and GAR-806 which gave a 0.527 kb product. After amplification 20 µl of PCR products were electrophoresed (Fig. 42, 43 and 45) and the remaining were dotted on nylon membrane and hybridized with probes GA153 (Fig. 44) and GA527 (Fig. 46). Fig. 42 and 43 showed gel electrophoresis of PCR products obtained from primers GAL-301 and GAR-432 which could detect 1 and 6 cells per reaction tube, respectively (lane 8 in both Figures). The calculation was based on the number of cells present in 20 µl of PCR reaction which was used for gel electrophoreses. Fig. 45 shows gel electrophoresis of PCR products obtained from primer GAL-301 and GAR-806 which could detect 6 cells per reaction tube. Fig. 44 and 46 show dot blot hybridization results of those PCR products which could be detected if only 1 to 6 cells were present in the PCR reaction mixtures which were used for hybridization. However, the results of some experiments showed that gel electrophoresis could detect the PCR products only if at least 10^2-10^3 cells were

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present per reaction by using 1/10 volume (15 µl) of 150 µl PCR products (the results not shown). This sensitivity could be brought down to 10^0 cell by dot blot hybridization with about 120 µl of the remainder of PCR products. Thus dot blot hybridization was used both to confirm the correct detection and to enhance the sensitivity of detection.

The sensitivity test for PCR method to detect *E. coli* in water and food were performed by using the same method with tenfold serial dilutions of *E. coli* artificially contaminated sample. An amplification product of *E. coli* by PCR method in water could be observed for at least eight bacteria per reaction by using two pairs of primers, primers GAL-301 with GAR-432 (Fig. 47) and primers GAL-301 with GAR-806 (Fig. 49). Dot blot hybridization with both probes, probe GA153 (Fig. 48) and GA527 (Fig. 50) also confirmed the detection of at least 8 cells per reaction. The results of PCR sensitivity tests for *E. coli* added to food by using primers GAL-301 with GAR-432 and probe GA153 shown in Fig. 51 and 52 and by using primers GAL-301 with GAR-806 and probe GA527 were shown in Fig. 53 and 54. The results indicated that as few as 7 CFU of *E. coli* present in the food sample were detectable.

From these sensitivity results, it was suggested that the PCR method from primers GAL-301 with GAR-432, primers GAL-301 with GAR-806, by using sample filtration through FHLP membrane, coupled with dot blot hybridization assay with probes GA153 and GA527, are capable of detection upto 1-10 cells per reaction tube (Fig. 42-54). Such cell number calculation was based on the cells present in the PCR product volumes which were solely used in gel electrophoresis or dot blot hybridization.

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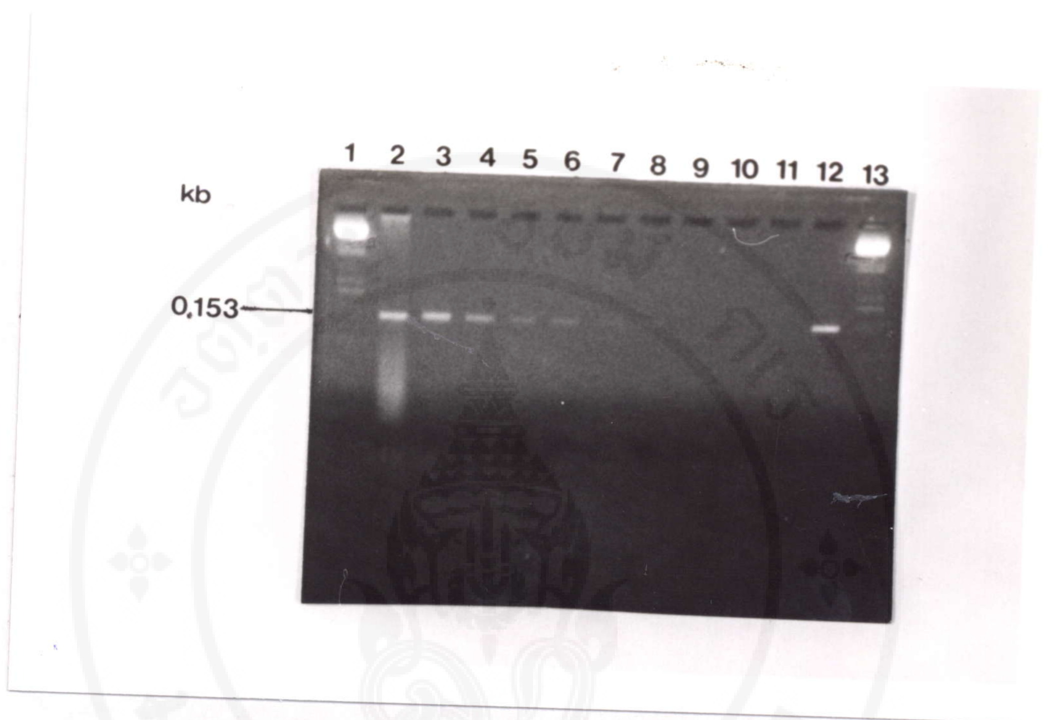

Fig. 42 Ethidium bromide-stained 4% Nusive (3:1) agarose gel of PCR amplified DNA from *uidA* gene of *E. coli*. Various numbers of *E. coli* cells in 0.85% normal saline were collected on Fluoropore FHLP membrane, lysed and PCR amplified with primers GAL-301 and GAR-432. Only 20 µl from 150 µl of total PCR products per reaction tube were loaded in each lane.

Lanes 1, 13. λ*Pst*I standard marker.

Lanes 2-10. PCR products from the serial dilution of *E. coli* K12 overnight culture for PCR amplification started from $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, 1x10, 1, 0.1 and 0 CFU/reaction tube (150 µl), respectively.

Lane 11. PCR reaction tube without *E. coli* chromosome.

Lane 12. PCR reaction tube with *E. coli* chromosome.

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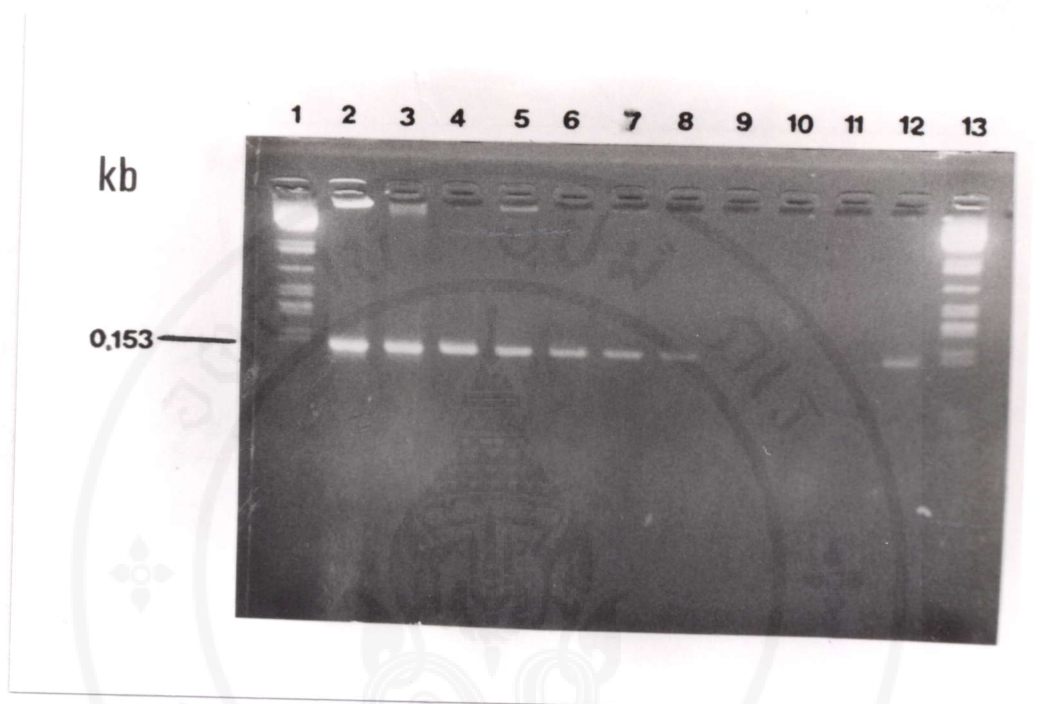

Fig. 43 Ethidium bromide-stained 4% Nusive (3:1) agarose gel of PCR amplified DNA from *uidA* gene of *E. coli*. Various numbers of *E. coli* cells in 0.85 % normal saline were collected on Fluoropore FHLP membrane, lysed and PCR amplified with primers GAL-301 and GAR-432. Only 20 µl from 150 µl of total PCR products per reaction tube were loaded in each lane.

Lanes 1, 13. λ*Pst*I standard marker.

Lanes 2-10. PCR products from the serial dilution of *E. coli* K12 overnight culture for PCR amplification started from $6x10^6$, $6x10^5$, $6x10^4$, $6x10^3$, $6x10^2$, 6x10, 6, 0.6 and 0 CFU/reaction tube (150 µl), respectively.

Lane 11. PCR reaction tube without *E. coli* chromosome.

Lane 12. PCR reaction tube with *E. coli* chromosome

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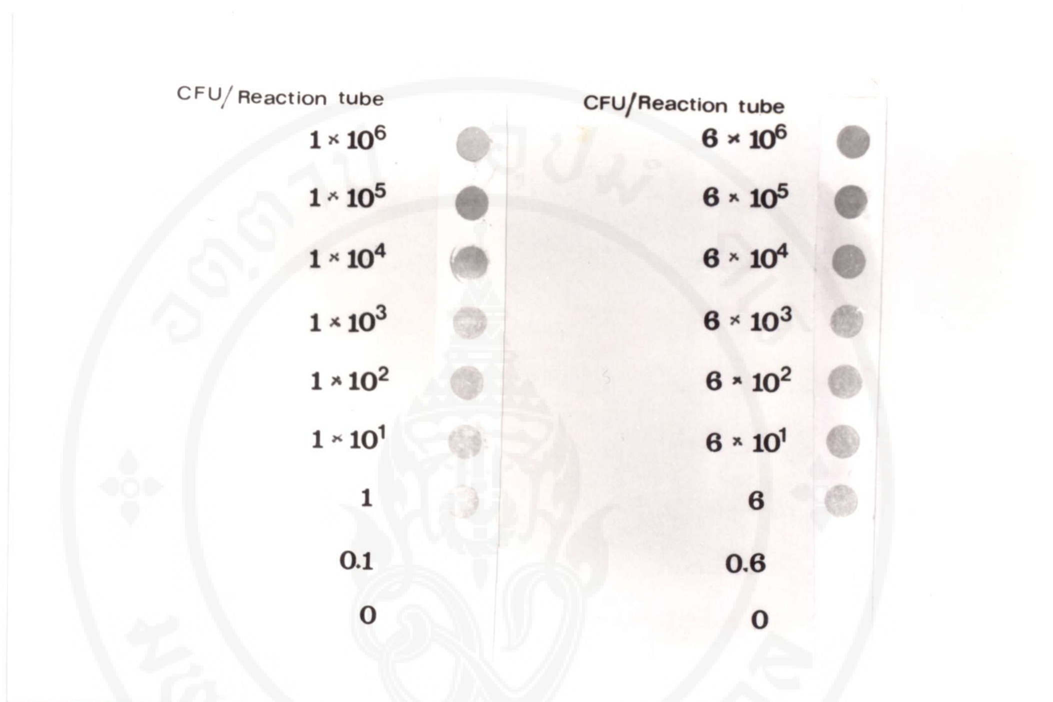

Fig. 44 Dot blot hybridization analysis after PCR amplification of serial dilution of overnight *E. coli* culture in 0.85% normal saline, using primers GAL-301 with GAR-432 and probe GA153. About 120 μl out of 150 μl PCR products were spotted in each dot. As few as one (left) and six (right) viable cells present in each PCR mixture (150 μl) were detectable.

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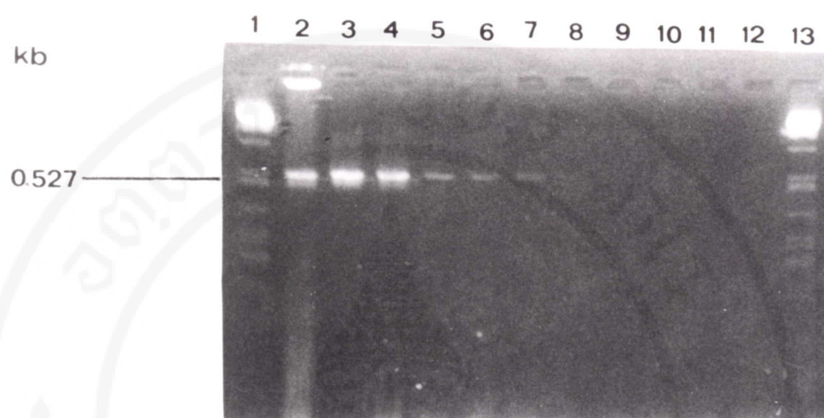

Fig. 45 Ethidium bromide-stained 4% Nusive (3:1) agarose gel of PCR amplified DNA from *uidA* gene of *E. coli*. Various numbers of *E. coli* cells in 0.85% normal saline were collected on Fluoropore FHLP membrane, lysed and PCR amplified with primers GAL-301 and GAR-806. Only 20 µl from 150 µl of total PCR products per reaction tube were loaded in each lane.

Lanes 1, 13. λ*Pst*I standard marker.

Lanes 2-10. PCR products from the serial dilution of *E. coli* K12 overnight culture for PCR amplification started from $6x10^6$, $6x10^5$, $6x10^4$, $6x10^3$, $6x10^2$, 6x10, 6, 0.6 and 0 CFU/reaction tube (150 µl), respectively.

Lane 11, 12. PCR reaction tubes without *E. coli* chromosome.

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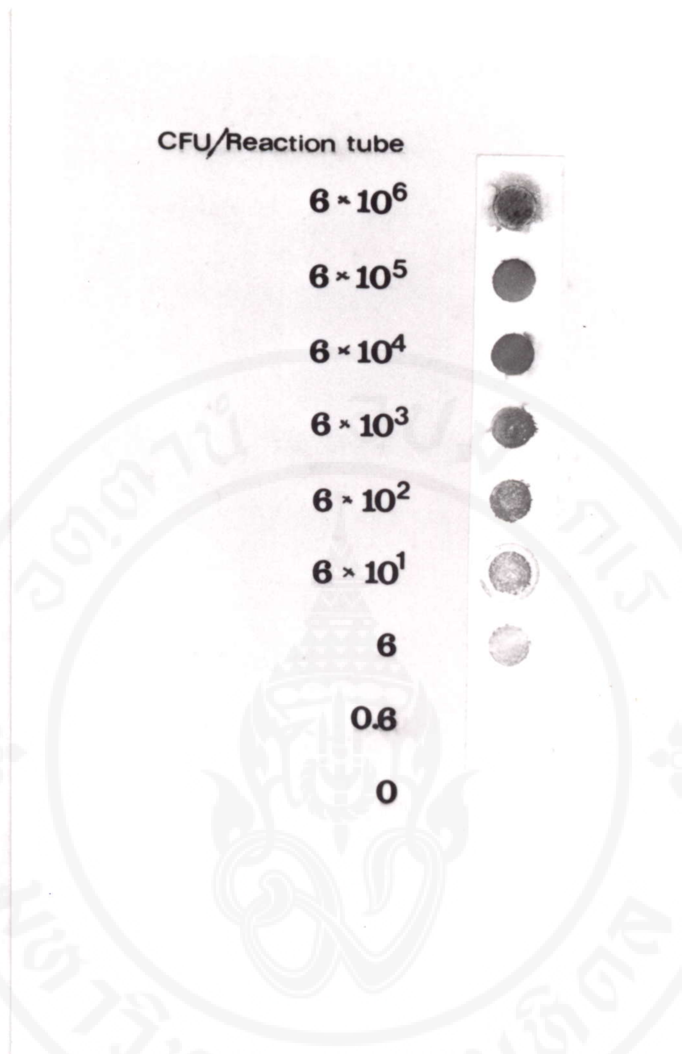

Fig. 46 Dot blot hybridization analysis after PCR amplification of serial dilution of overnight *E. coli* culture in 0.85% normal saline, using primers GAL-301 with GAR-806 and probe GA527. About 120 μl out of 150 μl PCR products were spotted in each dot. As few as six viable cells present in each PCR reaction mixture (150 μl) were detectable.

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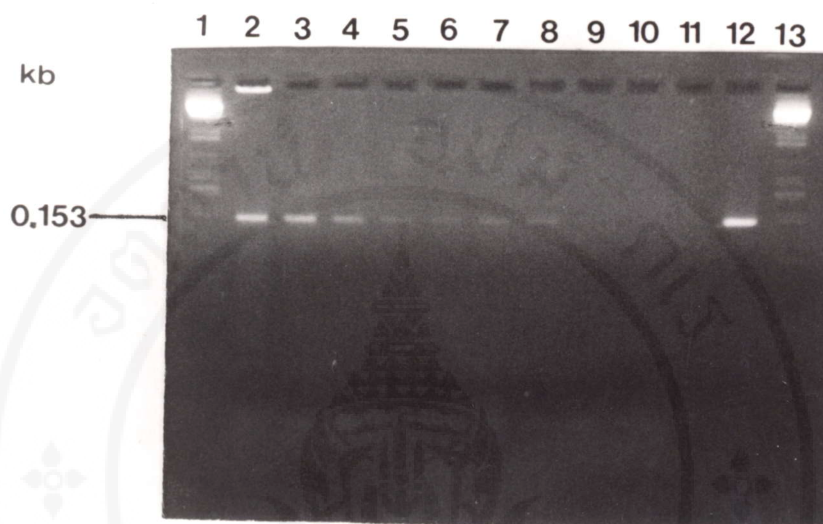

Fig. 47 Ethidium bromide-stained 4% Nusive (3:1) agarose gel of PCR amplified DNA from *uidA* gene of *E. coli*. Various numbers of *E. coli* cells in autoclaved distilled water were collected on Fluoropore FHLP membrane, lysed and PCR amplified with primers GAL-301 and GAR-432. Only 20 µl from 150 µl of total PCR products per reaction tube were loaded in each lane.

Lanes 1, 13. λ*Pst*I standard marker.

Lanes 2-10. PCR products from the serial dilution of *E. coli* K12 overnight culture for PCR amplification started from $8x10^6$, $8x10^5$, $8x10^4$, $8x10^3$, $8x10^2$, 8x10, 8, 0.8 and 0 CFU/reaction tube (150 µl), respectively.

Lane 11. PCR reaction tube without *E. coli* chromosome.

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Lane 12. PCR reaction tube with *E. coli* chromosome.

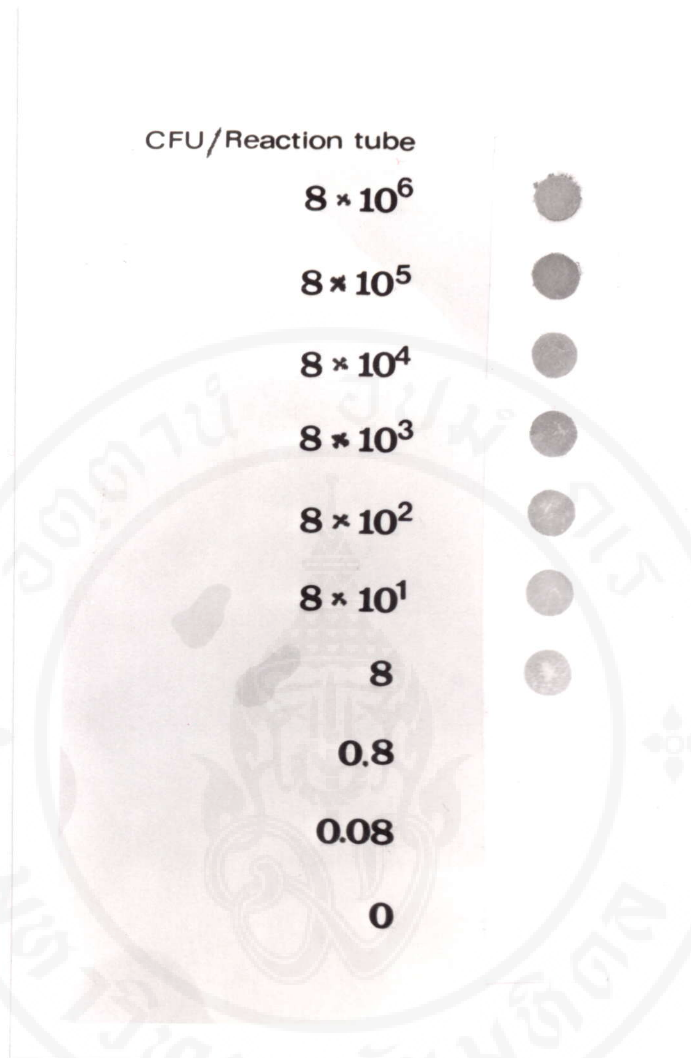

Fig. 48 Dot blot hybridization analysis after PCR amplification of serial dilution of overnight *E. coli* culture in autoclaved distilled water, using primers GAL-301 with GAR-432 and probe GA153. About 120 μl out of 150 μl PCR products were spotted in each dot. As few as eight viable cells in each PCR reaction mixture (150 μl) were detectable.

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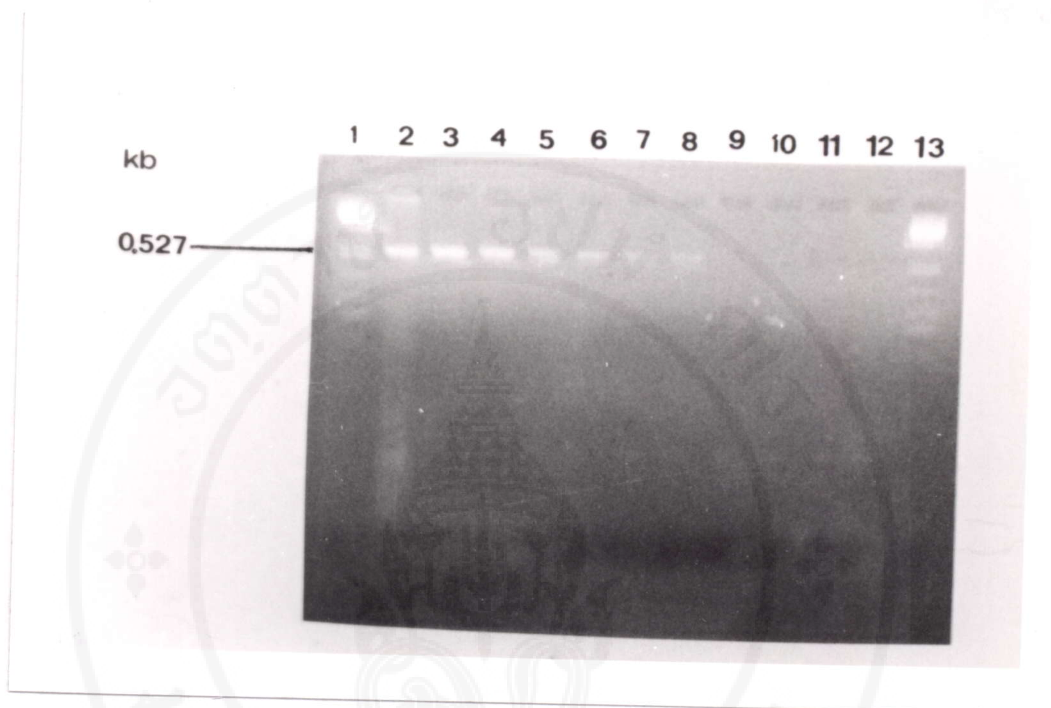

Fig. 49 Ethidium bromide-stained 4% Nusive (3:1) agarose gel of PCR amplified DNA from *uidA* gene of *E. coli*. Various numbers of *E. coli* cells in autoclaved distilled water were collected on Fluoropore FHLP membrane, lysed and PCR amplified with primers GAL-301 and GAR-806. Only 20 μl from 150 μl of total PCR products per reaction tube were loaded in each lane.

Lanes 1, 13. λ*Pst*I standard marker.

Lanes 2,-10. PCR products from the serial dilution of *E. coli* K12 overnight culture for PCR amplification started from 8×10^6, 8×10^5, 8×10^4, 8×10^3, 8×10^2, 8x10, 8, 0.8 and 0 CFU/reaction tube (150 μl), respectively.

Lane 11, 12. PCR reaction tubes without *E. coli* chromosome.

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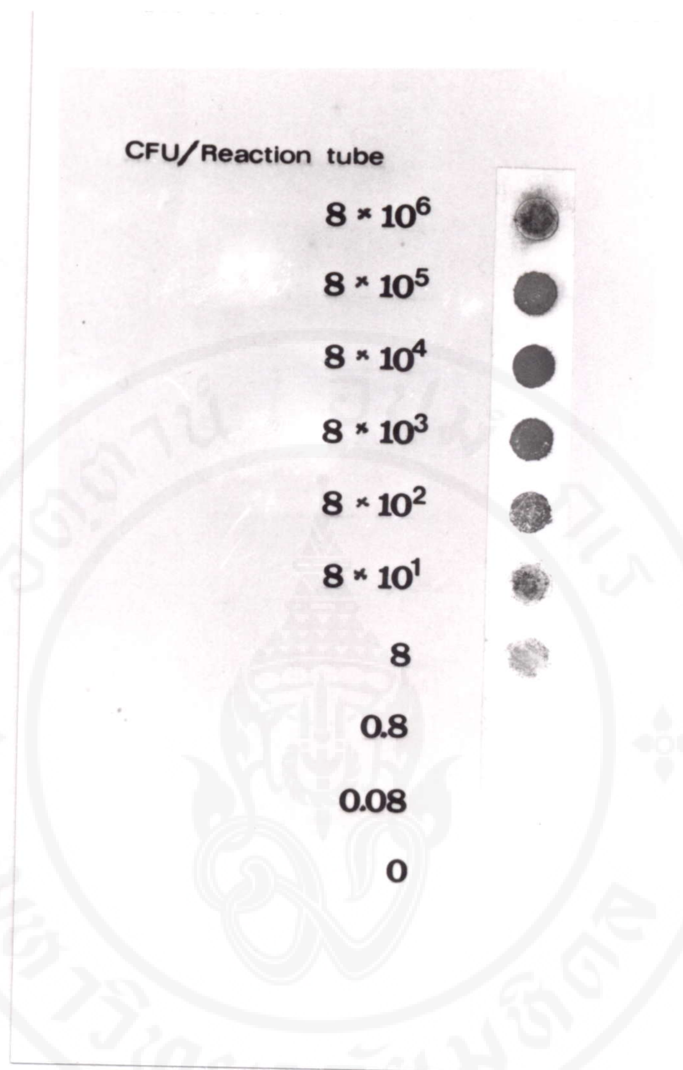

Fig. 50 Dot blot hybridization analysis after PCR amplification of serial dilution of overnight *E. coli* culture in autoclaved distilled water, using primers GAL-301 with GAR-806 and probe GA527. About 120 μl out of 150 μl PCR products were spotted in each dot. As few as eight viable cells in each PCR reaction mixture (150 μl) were detectable.

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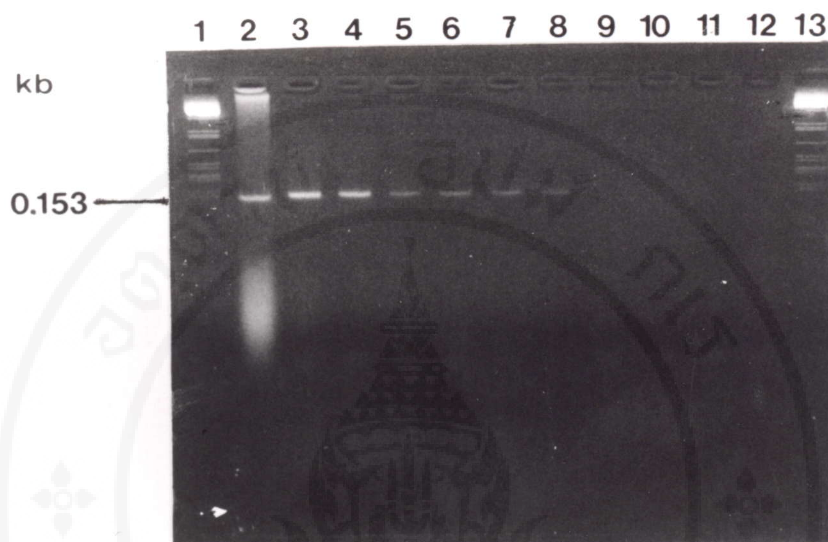

Fig. 51 Ethidium bromide-stained 4% Nusive (3:1) agarose gel of PCR amplified DNA from *uidA* gene of *E. coli*. Various numbers of *E. coli* cells added to food sample were collected on Fluoropore FHLP membrane, lysed and PCR amplified with primers GAL-301 and GAR-432. Only 20 μl from 150 μl of total PCR products per reaction tube were loaded in each lane.

Lanes 1, 13. λ*Pst*I standard marker.

Lanes 2,-10. PCR products from the serial dilution of *E. coli* K12 overnight culture for PCR amplification started from $7x10^6$, $7x10^5$, $7x10^4$, $7x10^3$, $7x10^2$, 7x10, 7, 0.7 and 0 CFU/reaction tube (150 μl), respectively.

Lane 11, 12. PCR reaction tube without *E. coli* chromosome.

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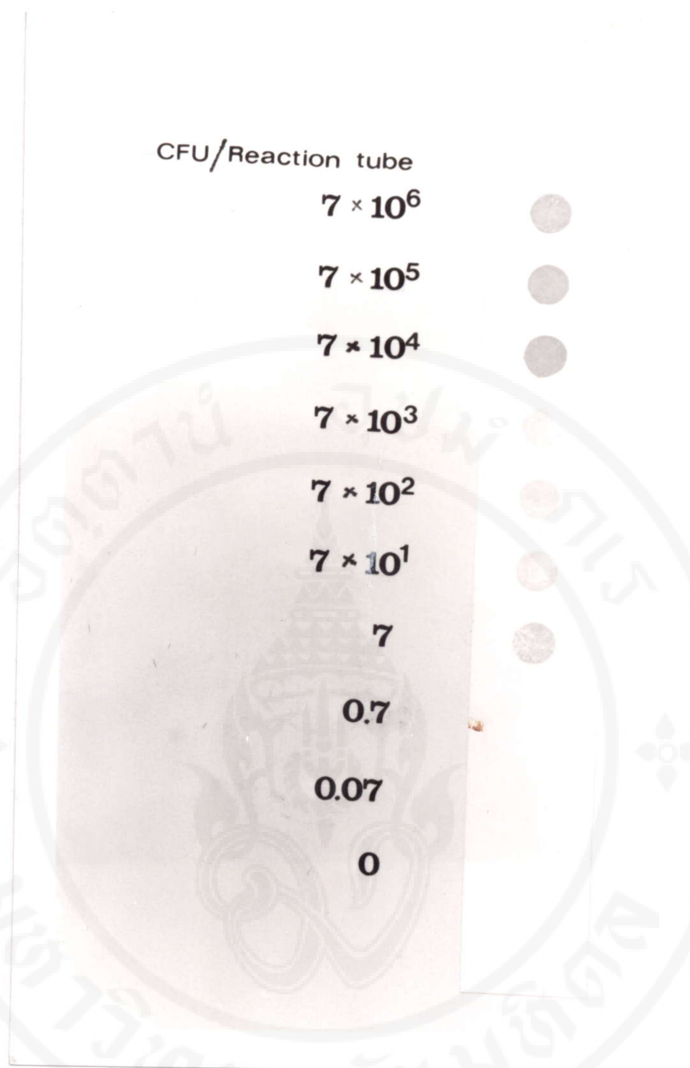

Fig. 52 Dot blot hybridization analysis after PCR amplification of serial dilution of overnight *E. coli* culture added to food, using primers GAL-301 with GAR-432 and probe GA153. About 120 μl out of 150 μl PCR products were spotted in each dot. As few as seven viable cells in each PCR reaction mixture (150 μl) were detectable.

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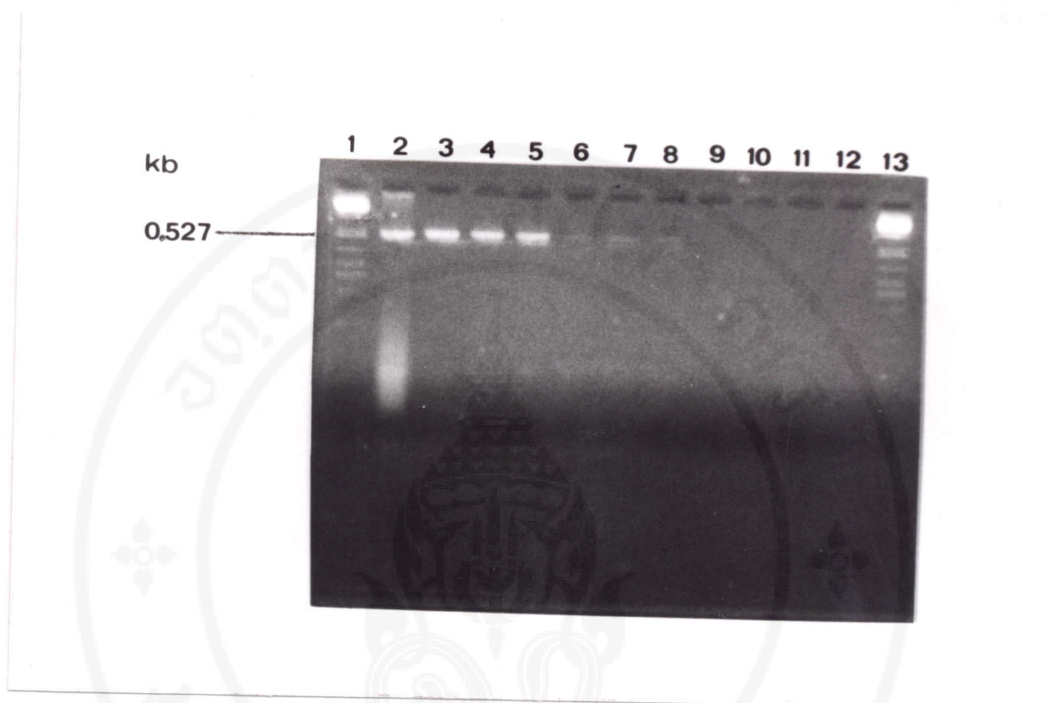

Fig. 53 Ethidium bromide-stained 4% Nusive (3:1) agarose gel of PCR amplified DNA from *uidA* gene of *E. coli*. Various numbers of *E. coli* cells added to food sample were collected on Fluoropore FHLP membrane, lysed and PCR amplified with primers GAL-301 and GAR-806. Only 20 μl from 150 μl of total PCR products per reaction tube were loaded in each lane.

Lanes 1, 13. λ*Pst*I standard marker.

Lanes 2,-10. PCR products from the serial dilution of *E. coli* K12 overnight culture for PCR amplification started from $7x10^6$, $7x10^5$, $7x10^4$, $7x10^3$, $7x10^2$, 7x10, 7, 0.7 and 0 CFU/reaction tube (150 μl), respectively.

Lane 11, 12. PCR reaction tube without *E. coli* chromosome.

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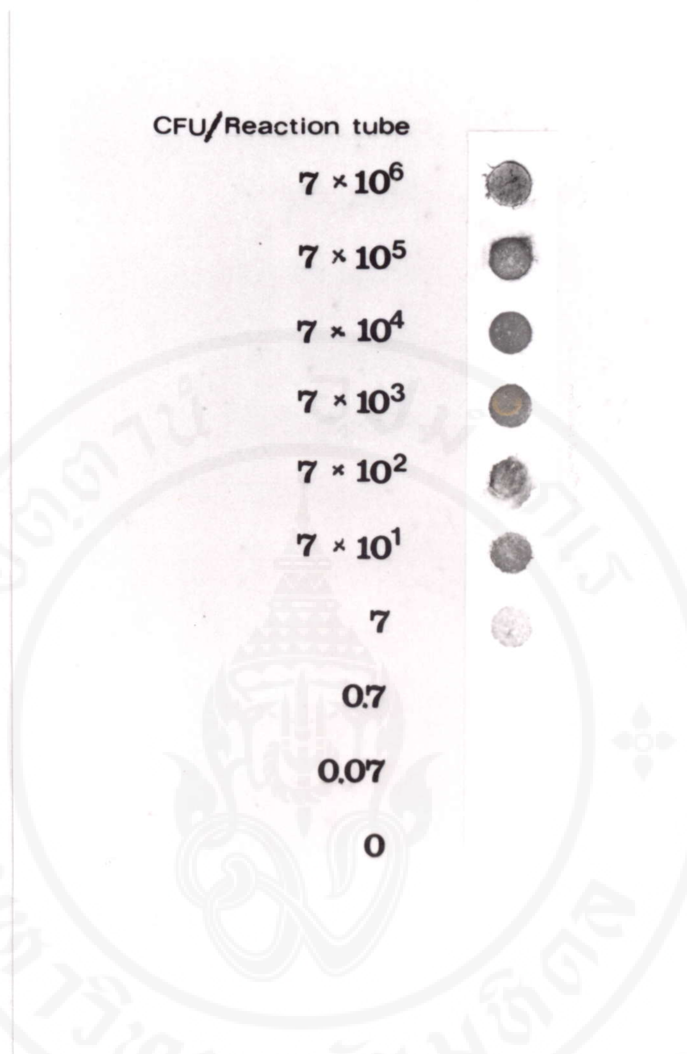

Fig. 54 Dot blot hybridization analysis after PCR amplification of serial dilution of overnight *E. coli* culture added to food, using primers GAL-301 with GAR-806 and probe GA527. About 120 µl out of 150 µl PCR products were spotted in each dot. As few as seven viable cells present in each PCR reaction mixture (150 µl) were detectable.

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CHAPTER V

DISCUSSION

Detection and enumeration of indicator bacteria is a primary important tool for monitoring of sanitary and microbiological quality of water and food. *E. coli* is a very important indicator which indicates feces contamination in water and food (Chang et al., 1989). In addition, some strains of *E. coli* are diarrheal disease such as Enterotoxigenic, Enteropathogenic, Enteroinvasive and Enterohemorrhagic (Levine M., 1987). Current conventional tests for *E. coli* are time consuming and lacking of both precision and specificity. Therefore the development of rapid methods which are highly specific for detection of *E. coli* have been performed. In order to develop method for monitoring presence of *E. coli* in water and food, DNA hybridization and PCR technique as detection method were studied. Based on the previous information for *E. coli* detection (Bej et al., 1991a, b, c, Cleuziat et al., 1990, Feng et al., 1991 and Green et al., 1991), a portion of the *uidA* gene of *E. coli* was selected to serve as the target for *E. coli* detection in this study. The *uidA* gene encodes for enzyme β-glucuronidase (GUS).

For DNA hybridization procedure, four specific DNA probes were tested for specificity and sensitivity. Two DNA probes, GA 153 and GA 527, derived from 0.153 kb and a 0.527 kb PCR products which obtained by PCR amplification of the part of *E. coli uidA* gene using two groups of primers, GAL-301 with GAR-432 and GAL-301 with GAR-806, respectively. The other two DNA probes, GA 175 and GA 549, derived from 0.175 kb and a 0.549 kb *Bam*HI/*Bst*XI restriction fragments from

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plasmids pGA1 and pGA2, respectively. These two plasmids were constructed by cloning of a 0.153 kb and a 0.527 kb PCR products using pGEM7 as vectors. The cloning was performed in order to use the cloned fragments as DNA probes. The hybridization results of these four digoxigenin labeled probes to *E. coli* and various enteric bacteria were similar. All 120 strains of *E. coli* which included 11 strains of Enteropathogenic *E. coli* (EPEC) and 14 strains of Enterotoxigenic *E. coli* (ETEC) were reactive with these probes. In addition all 11 *Shigella* strains tested in this study also showed the positive hybridization result. No reaction with the probes were observed in other *Enterobacteriaceae* including *Citrobacter*, *Enterobacter*, *Edwardsiella*, *Klebsiella*, *Morganella*, *Proteus*, *Pseudomomas* and *Salmonella* and other gram positive bacteria tested in this study including *Bacillus* and *Staphylococcus*.

When these hybridization results were compared with result obtained from GUS activity detection using microtiter plate assay, it was found that all 120 strains of *E. coli* (100%) and 11 strains of *Shigella* (100%) tested were reactive with the *uidA* gene probes even though four strains of *E. coli* (3.3%) which included one strain of EPEC and six strains of *Shigella* (54.6%) did not produce GUS. These results indicated the presence of DNA sequences of the *uidA* gene in all strains of *E. coli* and also *Shigella* tested including GUS negative strains. Factors which might affect GUS will be discussed later. Since the results of hybridization obtained with the four probes gave no difference observation, that is no cross hybridization was observed with other enteric bacteria. Therefore the extra 22 bp which came from the multicloning site of pGEM7 in probes GA 175 and GA 549 did not cause any cross hybridization. Hence,

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the GA175 and GA549 obtained from plasmid pGA1 and pGA2 can be used as the alternative source of the specific *uidA* probes to detect *E. coli* in addition to the probe derived from the PCR products.

The extreme specificity of *uidA* genes for *E. coli* or *Shigella* detection was confirmed by DNA amplification of *uidA* gene region in addition to its use as DNA probes. All of the *E. coli* and *Shigella* strains tested regardless of GUS activity were found to give product of expected size, a 0.153 and a 0.527 kb by using two set of primers , GAL-301 with GAR-432 or GAL-301 with GAR-806, respectively. Southern blot hybridization analysis with the probe GA 153 and GA 527 confirmed that all amplified DNA products were from *uidA* gene. The primers used are clearly specific for detection of *E. coli* and *Shigella*, independent of GUS phenotype and also implied that the *uidA* gene sequence was present in both *E. coli* and *Shigella*. It was also suggested that detection method based on the *uidA* gene in chromosomal region could not be used to distinguish *E. coli* from *Shigella* species. The observed results were in accordance to reports of other authors who have already pointed out the difficulty to differentiate these organisms by using genetic criteria (Spiering et al., 1989). In this detection test using PCR amplification method, direct PCR amplification from intact bacterial cells according to the method of Joshi et al.,(1991) was absolutely successful. This method was processed by growing bacteria in LB broth and then bacteria cells were lysed by heating at 100°C for 5 min and immediately cooled in ice water. The samples were then directly amplified by PCR without DNA isolation steps. Therefore using this method, the step of extraction and purification of DNA can be omitted.

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According to Feng et al.,(1991) who suggested that most *E. coli* carry *uidA* gene sequences as observed by the hybridization data, then the absence of GUS enzyme activity in some *E. coli* may be attributed to physiological factors or genetic differences. GUS is an inducible enzyme, and there is evidence that its expression is affected by lactose-induced catabolite repression (Feng et al., 1991). Genetic mutations in the regulatory gene for *uidA* gene or structural regions of *uidA* gene itself can also affect *uidA* expression or the production of nonfunctional GUS enzymes. The results obtained from DNA hybridization using part of *uidA* gene as a probe, could hybridize to both GUS positive and negative strain, therefore, the presence of at least *uidA* gene portion in all *E. coli* and *Shigella* strains was evidenced. The presence of *uidA* gene was also confirmed by PCR amplification of *uidA* gene portion which that both GUS positive or negative strains of *E. coli* and *Shigella* when amplified, gave the same size of PCR products in correspond to both pairs of primers. Therefore, it can be concluded that all *E. coli* and *Shigella* used in this study contained at least portion of *uidA* gene, that could be detected by either DNA hybridization or PCR techniques using indicated probes or primers, respectively. However, whether the nucleotide sequences of such portion are complete homology or not required, sequencing of *uidA* gene from both GUS-positive and negative isolates which may explain the cause of absence of GUS enzyme activity in some *E. coli* and *Shigella* despite of the presence of *uidA* gene. It is possible that the *uidA* gene in GUS negative strains might contain some point mutation. Therefore the methods for detection of *E. coli* that depended on detection of GUS activity may fail to detect some GUS-negative *E. coli* which may included phatogenic strains.

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Since *uidA* gene can serve as a target for PCR and gene-probe detection, sensitivity tests were performed to determine the minimum number of *E. coli* cells which could be detected by both procedures, dot blot hybridization and PCR amplification. Sensitivity of dot blot hybridization techniques with digoxigenin labeled DNA probes from *uidA* gene, probes GA 175 and GA 549 could detect at least 10^5 *E. coli* cells per dot. This sensitivity of *E. coli* detection is equal in all three conditions, *E. coli* diluted with either normal saline, autoclaved distilled water or *E. coli* that was artificially contaminated to octopus sample with serial dilution of *E. coli* could be detected at minimum of 10^5 CFU/dot. The sensitivity of detection could be brought down to 1-10 bacteria by using PCR. The experiments were performed by doing PCR directly from samples that were filtered though filter membrane. Filtration method was used to concentrate microorganisms from sample on the filter. The filters with microorganisms attached on it were subjected to PCR experiment. The filter used in this study was Fluoropore filters FHLP (Millipore) which was previously reported that it did not inhibit DNA amplification by PCR (Bej et al., 1991c and Oyofo et al., 1993). The sensitivity of bacterial detection by gel electrophoresis using only 1/10 volume (15 µl) of PCR product (150 µl) in some experiment (the results not shown) exhibited the sensitivity of detection only 10^2-10^3 cells while PCR incombination with dot blot hybridization always showed the ability of detection of 1-10 cells. These results implied that to ensure the ability of detection at least 10^2-10^3 cells should be present to generate PCR products that could be seen clearly by gel electrophoresis and if hybridization method was used to detect PCR products, only 1-10 cells was enough for PCR performance to generate the products that could be

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detected easily by hybridization. Similar level of sensitivity was also obtained by Bej et al. (1991) and Cleuziat et al. (1990). Bej et al. were able to detect one to two viable cells of *E. coli* by using the primers and radiolabeld probe which amplified and detected *uidR* gene of *E. coli*. PCR sensitivity for *E. coli* detection determined by Cleuziat et al. was at least eight bacteria per reaction.

This PCR process was also used to detect *E. coli* directly in drinking water sample. The ten brands of drinking water obtained from the shops in the canteen of Faculty of Science, Mahidol University and Ramathibodi Hospital were examined by three procedures, viable plate count, membrane filter culture technique on Fluorocult ECD agar and PCR amplification. Viable plate count was performed to check the amount of viable bacterial cells together with observing bacterial fluorescence colony which may be *E. coli* by using Fluorocult plate count agar (Merk) which has MUG in its composition. Membrane filter culture technique on Fluorocult ECD agar (Merk) was performed according to manufacturer instruction to detect *E. coli* colony which appeared as fluorescence colony under UV light and showed the indole positive reaction. PCR amplification was performed to detect *E. coli* by using both two sets of primers, GAL-301 with GAR-432 and GAL-301 with GAR-806. *E. coli* was not detected in the 10 water samples tested in this study by either pour plate, filtration culture and PCR amplification techniques though 8 out of 10 samples contaminated with large number of viable cells of other unidentified bacteria (10^3-10^4 cells per ml). Since the standard method to detect *E. coli* in drinking water should use at least 100 ml of water sample, the problem in detection of *E. coli* by using PCR from a 100 ml water sample filtered on a small diameter membrane (13 mm) was encounted

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especially in the sample with high microorganisms contamination. The problem was that water sample flowed through such small filter very slowly and the whole 100 ml of water sample could not be filtered on one such small size of FHLP membrane since the membrane pores were obstructed. Therefore, filtration of water samples which contain large number of other bacteria in the sample through FHLP membrane is not practical. Only 2 water samples with a volume of 100 ml could be filtered once through one FHLP membrane since the samples contained no bacterial cells. Therefore, it is suggested that in case of more turbid water samples, it may be necessary to use larger-diameter filter and requiring larger volumes of PCR mixture which however, not economical practical.

For detection of *E. coli* in food sample, frozen boiled octopus obtained from Export Promotion Section, Division of Food for Export Analysis, Ministry of Public Health was used for testing. The total aerobic microbial count of this sample revealed the presence of bacterial cells of 8.8×10^3 CFU/g which were not *E. coli* as identified by Multiple-tube fermentation technique using Fluorocult Lauryl Sulfate Broth (Merk). No fluorescence occurred under UV light and no indole reaction in the food homogenate and its all dilutions were observed as compared with positive control and negative control. Therefore, to monitor PCR technique in the food sample, artificially contaminated sample will be used.

The PCR amplification result of *E. coli* contaminated food sample showed that the longer the incubation time before performing PCR, the larger the amount of amplified DNA. This result indicated that the preenrichment step could enhance the sensitivity of detection if the sample contaminated with viable cells. Therefore very

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low number of *E. coli* (1-10 cells) contaminated in food sample could be detected by using PCR amplification and more PCR products could be produced if preenrichment step was used. Increasing of PCR product from samples with preenrichment step suggests that such PCR products were derived from living cells. The two procedures used in this study gave no difference results in sensitivity of detection. However, the second procedure which processed by direct PCR amplification with 10 μl of boiled homogenate from food sample is more practical than the first procedure that required processing of the sample by filtration through the FHLP membrane. The first procedure may be suitable for using with water sample and/or clear and very low tissue content food sample. In case of the second procedure, it seem to be more effective if this method was used in coupling with preenrichment step. Preenrichment step is especially required for obtimal PCR detection if the food sample contaminated with low number of *E. coli*. With preenrichment steps, more PCR products were produced, therefore the DNA bands in gel electrophoresis will be clearly seen and dot blot hybridization will not be necessarily performed. Moreover the procedures used in this study can save the time for DNA extraction or DNA purification from the sample before PCR amplification process. This will ease the method of PCR and shorten the time for bacterial detection.

An additional experiment was performed to study the possibility of PCR technique for detection of free DNA released from nonviable cells contaminated in food sample. A 1 ml of *E. coli* free food samples were mixed with 1 ng of *E. coli* K12 chromosomal DNA which is equivalent to DNA from 10^6 bacterial cells. The mixtures were then incubated at 37°C for 0, 30 and 60 min. These samples were analysed by

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PCR amplification using two procedures of sample filtration through FHLP membrane and sample centrifugation techniques. These two procedures gave no different results which indicated that PCR technique could amplify 1 ng of *E. coli* chromosomal DNA contaminated in food sample even after incubation at 37°C for 0, 30 and 60 min (Fig. 37 and 38). Therefore, the results indicated that if the food sample contaminated with dead cells or chromosomal DNA of *E. coli*, the PCR technique still could detect the DNA and gave rise to PCR products. This result is similar to the study of Josephson et al.,(1993) which showed that PCR methods can detect nonviable cells as long as intact target nucleic acid sequences are available. They illustrated that PCR can detect boiled *E. coli* cells in an environmental water sample which stored at 4°C for 2 weeks. Normally, once a cell dies in an environmental sample, its nucleic acids would be expected to be rapidly absorbed by colloidal material (Ogram et al., 1987), and such sorbed DNA can subsequently become available for PCR amplifications or act as a transforming agent (Khanna et al., 1990). The rate of degradation of nucleic acids will be dependent on the specific environmental sample. Hence, there will be a window of opportunity for false positives resulting from PCR amplifications of intact nucleic acids released from dead cells prior to degradation (Josephson et al., 1993). The application of PCR method must be taken with care in interpreting results and some mechanism for discriminating between viable and non-viable cells such as preenrichment step should be used. Moreover, a greater number and various kind of food samples will have to be analysed to establish the efficacy of the PCR method for food quality monitoring before it can be proposed as an alternative method for bacterial detection. Any different food samples may contain inhibitory substances

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which could inhibit PCR. The other factor which must be considerd is that the standard allowance of the amount of *E. coli* in each food is difference depending on types of food. Therefore PCR technique for *E. coli* detection should be used in food or water samples in which no *E. coli* is allowed to be present.

This study has developed the method to detect *E. coli* in water and food by using nonisotopic gene probe detection, direct PCR and PCR techniques by using filter-concentrated samples which modified from the previous studies (Bej et al., 1991a, b, c and Oyofo et al., 1993). Using these procedures, we can save the time (complete detection within 1-2 days), can test conveniently with several samples at the same time and especially can protect consumers from potential faecal contamination events because of its high specificity. Though there is cross reaction with *Shigella* , it is not the problem because *Shigella* is the pathogen which is not allowed to be present in water and food. However the cost of this method is an interesting factor which should be considered. Table 9 showed the estimate cost of chemicals and materials per 1 reaction of PCR and dot blot hybridization methods for detection of *E. coli* in food sample and Table 10 showed the estimate cost of materials used in each technique. The calculation of cost excluded the cost of investment of instrument, labor cost, water and electricity supplies. The cost for PCR method includes the cost of *Taq* polymerase and dNTPs of each company (Boehringer mannheim and Perkin-Elmer Cetus), primers, FHLP membrane, mineral oil, stomacher bag, LB-broth, gel electrophoresis, tip and PCR reaction tube. The cost for dot blot hybridization include the cost of Dig DNA labeling and detection kit, nylon membrane, buffers, stomacher bag, and LB broth. The cost of dot blot hybridization in Table 10 was calculated on the basis of 96

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samples performed at the same time. In the case of bacterial detection in water, the method used is only PCR by using filter-concentrated sample and the cost showed in Table 9 must be subtracted with 28.23 bath which is the cost of stomacher bag and LB broth. If *Taq* polymerase can be produced by individual laboratory, the price will be about 37.50-75.00 baht cheaper than the value in Table 9. These costs are still expensive. Thus, the development of the alternative methodology to reduce cost and provide an effective tool which is faster and cheaper than the conventional viable culture method is required. Table 11 showed comparison of some categories between 4 methods used for *E. coli* detection in food sample. The four methods were direct PCR amplification, PCR amplification from filter membrane, dot blot hybridization, and culture method.

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Table 9 Estimate cost for PCR and dot blot hybridization methods for detection of *E. coli* in 1 food sample[a].

Methods	Estimate cost per 1 reaction (Baht)
Direct PCR with *Taq* and dNTPs of Boehringer mannheim (BM)	115.32
Direct PCR with *Taq* and dNTPs of Perkin-Elmer Cetus (PEC)	134.92
PCR by using filter-concentrated samples with *Taq* and dNTPs of BM	208.03
PCR by using filter-concentrated samples with *Taq* and dNTPs of PEC	238.93
Dot blot hybridization	37.66

[a] The cost for PCR was calculated from PCR performance of 1 sample at a time, whereas the costs for dot blot hybridization per one sample was calculated from processing of 96 samples at a time.

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Table 10 Estimate cost of materials used in each method for detection of *E. coli* in food sample.

Materials	Direct PCR with *Taq* and dNTPs of BM (1 sample)	Direct PCR with *Taq* and dNTPs of PEC (1 sample)	PCR by using filter-concentrated samples with *Taq* and dNTPs of BM (1 sample)	PCR by using filter-concentrated samples with *Taq* and dNTPs of PEC (1 sample)	Dot blot hybridization (96 samples)
1.Stomacher bag	14	14	14	14	1344
2.LB broth for food suspension	14.23	14.23	14.23	14.23	1366.08
3.Taq polymerase	40	43	80	86	-
4.dNTPs	6.4	23	9.6	34.5	-
5.PCR buffer	0	0	0	0	-
6.Primers	2.68	2.68	4.03	4.03	-
7.FHLP membrane	-	-	48.15	48.15	-
8.Tip and tube for PCR	11.60	11.60	11.60	11.60	-
9.mineral oil	0.09	0.09	0.1	0.1	-
10.Gel electrophoresis	26.32	26.32	26.32	26.32	-
11.Nylon membrane	-	-	-	-	12.50
12.Reagent of Dig DNA labeling and detection kit (BM)	-	-	-	-	562.5
13.Buffers and solutions used for hybridization	-	-	-	-	330.69
Total (Baht)	115.32	134.92	208.03	238.93	3615.77

Note The estimated cost for dot blot hybridization for each sample was 37.66 Baht per sample if 96 samples were performed at the same time. If only one sample was used at a time, the calculation will be 260 Baht per sample.

-, The material was not used in this technique.

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Table 11 Comparison of the 4 methods used for *E. coli* detection in food sample.[a]

Categories	Techniques			
	Direct PCR amplification	PCR amplification from filter membrane	Dot blot hybridization	Culture method
Cost/reaction (Baht)	115.32 (BM) 134.92 (PEC)	208.03 (BM) 238.93 (PEC)	37.66 (Dig)	75.22 (Fluorocult media) 70.58 (Conventional media)
Time	4 [b] hrs	5 [b] hrs	2 days	1-2 days (Fluorocult media) 4-6 days (conventional media)
Specificity	100%[c]	100%[c]	100%[c]	94-97%[c] (Fluorocult media)
Sensitivity	1-10 cells	1-10 cells	10^5 cells	ND[d]

[a] The cost for PCR was calculated from PCR performance of 1 sample at a time, whereas the costs for dot blot hybridization per one sample was calculated from processing of 96 samples at a time.

[b] No preenrichment step but including a 1 hr gel electrophoresis.

[c] Percent of positive result with *E. coli*.

[d] Not determined.

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CHAPTER VI

SUMMARY

In this study DNA probe and PCR techniques are subjected to study for the possibility as an alternative method for *E. coli* detection. Four digoxigenin labeled DNA probes, GA153, GA527, GA175 and GA549, derived from *uidA* gene of *E. coli* K12 which encodes β-glucuronidase were tested for specificity and sensitivity. The probes GA153 and GA527 were the PCR products derived from amplification of *uidA* gene using primers GAL-301 with GAR-432 and primers GAL-301 with GAR-806, respectively. Subcloning of GA153 and GA527 in pGEM7 gave rise to plasmids pGA1 and pGA2. The probes GA175 and GA549 were obtained by cleaving pGA1 and pGA2, respectively with *Bam*HI and *Bst*XI. Hence, the 2 latter probes were similar to the probes GA153 and GA527 except that there were 22 basepairs from multicloning sites of pGEM7 in addition to DNA sequence derived from *uidA* gene. These four probes gave no different result in detection of *E. coli* or other enteric bacteria by dot blot hybridization technique. All 120 strains of *E. coli* tested gave positive result. Whereas other gram positive and *Enterobacteriaceae*, except, 11 strains of *Shigella*, could not hybridize with these probes. DNA amplification of *uidA* gene region using three primers, GAL-301, GAR-432 and GAR-806 showed that all *E. coli* and *Shigella* strains tested gave PCR products of expected size of a 0.153 and a 0.527 kb. Therefore the probes and primers used are highly specific for detection of *E. coli* and *Shigella*. Sensitivity of dot blot hybridization technique with these four probes could detect at least 10^5 *E. coli* per dot while PCR technique could detect 1-10

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E. coli cells present in the sample. Based on the sensitivity of detection, PCR amplification from filter-concentrated samples were an effective method to detect *E. coli* in water sample. The ten brands of drinking water samples were examined and the result showed that there are no *E. coli* in these samples. Detection of *E. coli* in food was also performed using the frozen boiled octopus. The food samples were mixed with *E. coli* cultures and the mixtures were subjected to PCR directly or after filtration through FHLP membrane. It was found that both procedures of PCR amplification, PCR amplification of samples concentrated on filter and direct PCR amplification of samples could detect *E. coli* which were artificially contaminated food samples. Direct PCR amplification of food samples without concentration of samples on FHLP membrane was found more practical and effective procedure. Though PCR amplification method was an effective and simple procedure with high specificity and sensitivity, precaution of its practical use should be concerned. Since, mixing the food samples with *E. coli* chromosomal DNA and subjected to PCR amplification could also produce the PCR products even the samples were incubated at 37°C, for 30 and 60 min prior to PCR performance. Therefore PCR amplification could also detect free DNA released from non viable cells. However, PCR amplification of food samples artificially contaminated with *E. coli* after preenrichment for 1, 3, and 6 hrs was performed and it was found that the method could differentiate viable and nonviable cells present in the food samples. The cost of PCR method was rather high, the development of effective PCR procedure with lower cost is thus still required.

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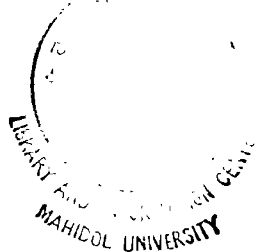

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