การตรวจหา enterotoxin gene โดยวิธี Polymerase Chain Reaction และการจำแนกยีโนทัยป์ของเชื้อ *Clostridium perfringens* โดยวิธี Pulsed – Field Gel Electrophoresis

นางสาวจิรวรรณ สรุปราษฎร์

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ENTEROTOXIN GENE DETECTION BY POLYMERASE CHAIN REACTION AND GENOTYPING OF *CLOSTRIDIUM PERFRINGENS* BY PULSED – FIELD GEL ELECTROPHORESIS

Miss Chirawan Saruprad

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จุฬาลงกรณ์มหาวิทยาลัย

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ตรวจหา enterotoxin gene ของเชื้อ *C. perfringens* ทั้งหมด 106 สายพันธุ์ โดยเป็นเชื้อที่แยกได้จากอาหารและน้ำ 80 สายพันธุ์ จากอุจจาระผู้ป่วยในโรงพยาบาล 20 สายพันธุ์ จากอุจจาระผู้ป่วยนอกโรงพยาบาล 1 สายพันธุ์ และเชื้อแยกได้จากอุจจาระของผู้ป่วยจากโรคอาหารเป็นพิษจากประเทศญี่ปุ่น 5 สายพันธุ์ โดยใช้วิธี polymerase chain reaction (PCR) ผลปรากฏว่า สายพันธุ์ที่แยกได้จากอาหารและน้ำ รวมทั้งอุจจาระผู้ป่วยในโรงพยาบาลไม่มี *cpe* gene ในขณะที่สายพันธุ์ที่แยกได้จากอุจจาระผู้ป่วยนอกโรงพยาบาลและสายพันธุ์ที่แยกได้จากอุจจาระผู้ป่วยโรคอาหารเป็นพิษจากประเทศญี่ปุ่น มี *cpe* gene

ส่วนการตรวจหา enterotoxin ของเชื้อโดยใช้วิธี reverse passive latex agglutination (RPLA) ให้ผลต่างจากการตรวจหา *cpe* gene โดยใช้วิธี PCR เนื่องจากวิธี RPLA ต้องการการสร้างสปอร์ ซึ่งเชื้อทั้งหมดไม่สามารถสร้างสปอร์ในสภาวะของห้องปฏิบัติการที่ใช้ในการศึกษาครั้งนี้

จำแนกเชื้อ *C. perfringens* ทั้งหมด ได้เป็น 88 pulsotypes โดยการใช้ pulsed – field gel electrophoresis (PFGE) แยกชิ้นส่วนของ โครโมโซม ซึ่งตัดด้วย *Sma* I เชื้อที่แยกได้จาก ผู้ป่วยนอกโรงพยาบาล 1 สายพันธุ์ เป็นเชื้อ pulsotype เดียวกับเชื้อที่ได้จากอุจจาระของผู้ป่วยด้วยโรคอาหารเป็นพิษจากประเทศญี่ปุ่น คือ pulsotype 85

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

ภาควิชา สหสาขาจุลชีววิทยาทางการแพทย์	ลายมือชื่อนิสิต ....
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Enterotoxin gene detection in 106 *Clostridium perfringens* isolates including 80 isolates from food and water, 20 isolates from stools of admitted patients with diarrhea, one isolate from stool of non –admitted patient with diarrhea and 5 isolates from food poisoning outbreaks in Japan was performed using polymerase chain reaction (PCR) technique. None of the isolates from food and water and from food and water and from stool of the admitted patients had enterotoxin gene while the isolate from stool of one non-admitted patient as well as all 5 food poisoning isolates from Japan had enterotoxin gene.

Reverse passive latex agglutination (RPLA) was also used to detect CPE production *in vitro*. It was indicated that *cpe* gene detection by PCR and RPLA did not provide correlated results in the detection of enterotoxigenic isolates. Thus, RPLA required *in vitro* sporulation and not all isolates could sporulate in the laboratory conditions used in this study.

All *C. perfringens* isolates were discriminated into 88 different pulsotypes using pulsed-field gel electrophoresis (PFGE) of the *Sma* I restricted chromosomal fragments. One isolate from non-admitted patient with diarrhea was in the same pulsotype as the two food poisoning isolates from Japan which was Pulsotype 85

Department......Inter-Department of Medical...... Student's signature..............................

Field of study.....Medical Microbiology........ Advisor's signature..............................

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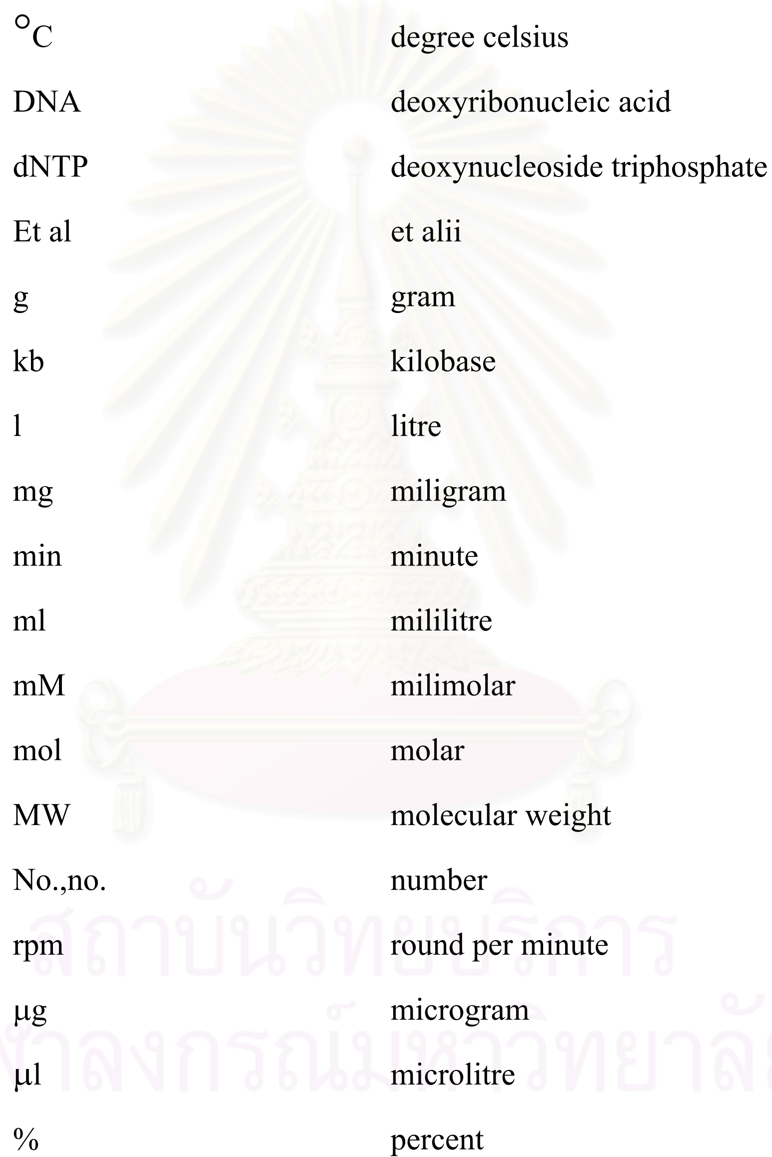
ABBREVIATIONS

°C	degree celsius
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
Et al	et alii
g	gram
kb	kilobase
l	litre
mg	miligram
min	minute
ml	mililitre
mM	milimolar
mol	molar
MW	molecular weight
No.,no.	number
rpm	round per minute
µg	microgram
µl	microlitre
%	percent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CHAPTER I

Introduction

Clostridium perfringens is a gram-positive spore-forming bacteria. The organism has been classified into five types (A through E) on the basis of its ability to produce the major lethal toxins (alpha, beta, epsilon and iota toxins) (1).

C. perfringens enterotoxin(CPE) produced in the intestine during sporulation is responsible for symptoms associated with the classical food poisoning caused by *C. perfringens* type A(2). Illness results from the ingestion of food contaminated with about 10^8 viable vegetative cells of enterotoxin-producing *C. perfringens*(3). The detection of *C. perfringens* in food indicated the faecal contamination(4). *C. perfringens* is ubiquitous, occurring in soil, water, sewage, and the intestinal tract of human(5).

The reports from the United States and elsewhere indicated that almost all outbreaks and cases of *C. perfringens* food-borne gastroenteritis were due to the type A strains. In *C. perfringens* type A food-borne disease, the food vehicle is almost always an improperly cooked meat or a meat product, such as gravy(3).

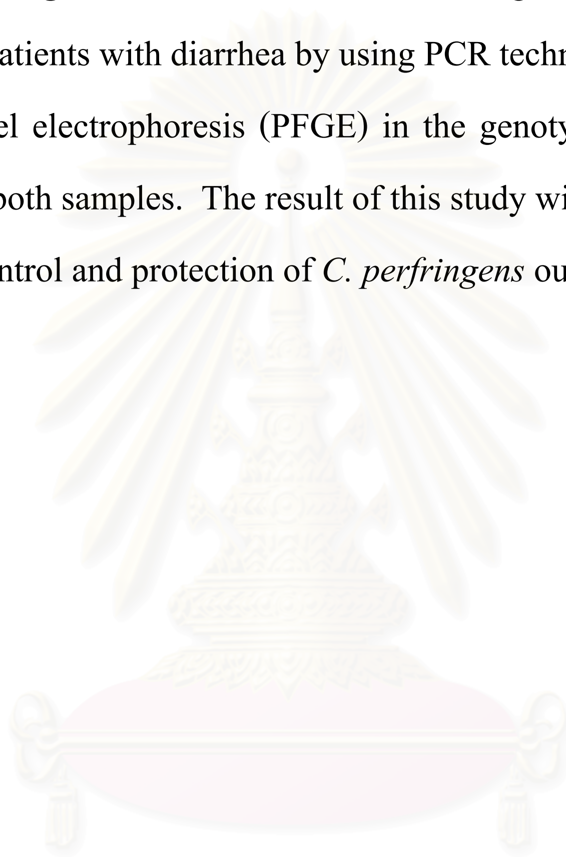
The Center for Disease Control and Prevention (CDC) during 1973 to 1987 reported that there were actually about 652,000 cases of *C. perfringens* type A food poisoning in the United States each year, with an average of 7.6 deaths per year and annual costs of the treatment were \$123 million(5). Because of this important health problem, reseachers in the developed

countries including America, Japan and those in Europe have interested in developing the methods for detection of the enterotoxigenicity of *C. perfringens* in foods and faeces Firstly, these methods were based on phenotypic characteristics of the organism such as reverse passive latex agglutination (RPLA), enzyme-linked immunosorbent assay (ELISA) and Vero cell assay(5). Later on, these methods have been shown to have some limitations because enterotoxin would be produced when the organism do sporulate only and many strains of *C. perfringens* do not sporulate well in the laboratory conditions(6). The false-negative results were often obtained(7).

Recently, enterotoxigenic *C.perfringens* stains have been successfully detected based on the genotypic characteristics. The use of polymerase chain reaction (PCR) to amplify the *cpe* gene has been recommended as the method of choice(6) after the gene encoding *C. perfringens* enterotoxin was sequenced by Damme-Jongsten in 1989(2). Daube (1994)(8) and Yoo (1997)(9) detected the enterotoxin gene of *C. perfringens* by using polymerase chain reaction method and indicated that this method showed higher sensitivity and high specificity more than the other methods and did not need the sporulation of organism in performing detection(9).

Pulsed-field gel electrophoresis (PFGE) has been shown to be the best method in the epidemiological study of *C. perfringens* The patterns of the restricted fragment of chromosomal DNA from PFGE have been used to discriminate *C. perfringens* isolates into different pulsotypes which could be use to indicate whether the isolates come from the same clone (outbreak strain) or not(10, 11, 12).

Despite the fact that food poisoning remains as the health problem in Thailand, we still do not have the data on the recovery of enterotoxin producing organism from food and faeces. The aims of the present study is to detect *C. perfringens* isolates with enterotoxin gene from food and from faeces of the patients with diarrhea by using PCR technique and using pulsed-field gel electrophoresis (PFGE) in the genotyping of *C. perfringens* isolated from both samples. The result of this study will be one of the primary data for the control and protection of *C. perfringens* outbreaks in Thailand.



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CHAPTER II

OBJECTIVES

1. To use polymerase chain reaction technique to detect enterotoxigenic *Clostridium perfringens* strains which were isolated from foods and stool specimens.
2. To use reverse passive latex agglutination assay (commercial kit : oxoid) to detect enterotoxin produced by *C . perfringens*
3. To genotype all *C. perfringens* isolates using pulsed-field gel electrophoresis (PFGE).

CHAPTER III

LITERATURE REVIEW

I. *Clostridium perfringens.*

General characteristics.

C. perfringens is a Gram-positive, rod-shaped, about 4x1.5μm encapsuled, nonmotile bacterium(13). Most strains of *C. perfringens* under conditions of rapid growth produce many very short forms. Spores are rarely seen in artificial culture. They are typically subterminal and not bulging. Many strains do not sporulate readily(14).

Cultural character.

The organism is not strict anaerobe, growing readily in the presence of small amounts of oxygen. The optimum temperature ranges from 37-45 $^{\circ}$C. The organism grows best on carbohydrate-containing media, e.g. glucose agar or glucose-blood agar. Surface colonies are circular, about 2-4 mm in diameter after 24 h. incubation, convex, semitranslucent, smooth and with an entire edge. On egg yolk agar *C. perfringens* produce diffuse opalescence, which is inhibited by *C. perfringen* antitoxin. Most strains of *C. perfringens* produce a double zone of hemolysis on blood agar plates(13). In ordinary milk medium rapid fermentation of the lactose occurs, with the subsequent development of a characteristic "stormy clot" reaction.

Some strains of *C. perfringens* type A cause a characteristic form of food poisoning, which differs from typical type A strains in the greater heat resistance of its spores, its feeble toxingenicity and it slight or non-haemolytic effect on blood agar, that these heat-resistant, non-haemolytic variants were not the only, and indeed not even the commonest, strains of *C. perfringens* that cause food poisoning in man, for both non-haemolytic non-heat resistant strains, and haemolytic non-heat resistant strains were incriminated in outbreaks of food poisoning both in the United Kingdom and in America(13, 14).

Biochemical characteristics.

All types ferment lactose. They are nagler's test positive and catalase, indole negative.

Natural Habitats (Reservoirs for *C. perfringens* type A Food poisoning).

C. perfringens is ubiquitous throughout the natural environment. This organism is commonly encountered in soils (at levels of 10^3 to 10^4 CFU/g), foods (e.g., approxinmately 50% of raw or frozen meat contain some *C. perfringens*), dust, and the intestinal tracts of human (e.g., human feces typically contain 10^3 to 10^6 *C. perfringens* cells per g)(5). This wider spread distribution of *C. perfringens* has long been considered the primary explanation for the prevalence of *C. perfringens* type A food poisoning.

Virulence factors.

The pathogenicity of the organism is associated with several toxins such as enterotoxin, alpha, beta, epsilon and iota toxin.

Enterotoxin (CPE)

C. perfringens enterotoxin (CPE) is considered to be the virulence factor responsible for the symptoms of *C. perfringens* type A food poisoning in human(15). The enterotoxin causes severe disturbance of metabolism and transportation of water and electrolytes in the intestine, and that the intestine treated with the enterotoxin showed serious histological damage including bleb formation of epithelial cells(16). From previous experimental results, it is clarified that direct cytotoxic action of the enterotoxin on intestinal epithelial cells is the cause of diarrhea which is the symptom of the food poisoning caused by *C. perfringens*.

CPE was first purified and characterized in the early 1970S(17); additional information about this protein was later supplied by amino acid and nucleotide sequencing studies(18-22). Collectively, these studies indicates that the CPE made by *C. perfringens* NCTC 8239 is a single, 319 amino acid polypeptide with a molecular weight of 35 kDa and an isoelectric point of 4.3(18). Nucleotide sequence analysis indicates that CPEs made by different *C. perfringens* strains share extensive amino acid homology, but may also have some limited microsequence variations(18, 22, 23). CPE does not share sequence homology with any known proteins,(18) except for antp 70/C1, one of the nonneurotoxic proteins found in the botulinum C1 neurotoxin gene comp(24). The significance, if any, of the limited homology between CPE and the Antp 70/C1 protein is unclear.

Physical studies suggest that the secondary structure of CPE consists of approximately 80% β-pleated sheet and 20% random coil(17). The tertiary structure of CPE currently remains undetermined owing to the difficulties in crystallizing the toxin for X-ray diffraction analysis. CPE's biological activity is both heat-labile, i.e., heating the toxin to $60^{\circ}C$ for 5 min cause an irreversible inactivation and pH labile, i.e., CPE's biological activity is lost at pH<5 or >10(25). However, the toxin is resistant to the treatment with some proteases; in fact, limited treatment of the purified enterotoxin with trypsin or chymotrypsin causes a two-to three-fold activation in CPE biological activity(26, 27). This activation effect has prompted suggestions that intestinal proteases may activate CPE during food poisoning(26).

In vivo Effects of *C. perfringens* Enterotoxin

A. CPE effects on the gastrointestinal system

The diarrheal symptoms associated with *C. perfringens* type A food poisoning provided the first indication that CPE was active on the GI tract(27). Subsequent studies with purified CPE have shown that this enterotoxin caused a rapid fluid and electrolyte loss from the GI tract in several different animal models(28-33). These effects can develop within a short period of time after the exposure to CPE. All regions of the small intestine respond to CPE treatment, although the ileum is particularly CPE sensitive(30, 33). Interestingly, while rabbit colonic cells bind CPE at high levels, the rabbit colon does not show significant effects when challenged with enterotoxin; it remains unclear how CPE affects the human large intestine.

The intestinal effects of CPE in animal models may be distinguished from those of cholera enterotoxin in several respects.

First, unlike cholera toxin, CPE does not effect small intertinal cAMP levels, but does inhibit small intestinal glucose absorbtion(30).

Second, cholera toxin does not cause visible tissue damage to the gastrointestinal tract while CPE produces extensive histopathological damage to the small intestine(30-34). CPE belongs to the class of "cytotoxic" enterotoxins, such as the C*lostridium difficile* toxins, on the basis of how quickly CPE-induced intestinal tissue damage develops : in animal models, histopathological damage is detectable within as little as 15-30 minutes of treament with purified CPE(32), while damage from other cytotoxic enterotoxins typically develops more slowly in these models(35).

In animal models, the initial CPE-induced histopathological damage is limited to the villi tips of the small intestine, but with time the entire small intestinal villus of CPE-treated animals eventually suffers extensive desquamation(31, 34).

Strong circumstantial evidence suggests that this histopathological damage plays an important role in producing the fluid and electrolyte losses that are associated with CPE treatment of the small intestine. The onsets of both fluid and electrolyte transport alterations have been shown to closely coincide with the development of histopathological damage in CPE-treated rabbit ileal loops(32).

Further, only those CPE doses able to cause histopathologic damage in rabbit ileal loops can also produce fluid and electrolyte loss in this model.(10).

CPE lethality

In addition to these intestinal effects, highly purified CPE rapidly kills animals when administered intravenously (the LD_{50} of CPE in mice is 50μg/kg)(36).

Animal studies have identified at least three pathophysiologic mechanisms that could potentially be involved in lethal effect;

(1) CPE may cause hyperpotassemia through its effects on the liver-this hyperpotassemia may, in turn, lead to sudden cardiac failure(36).

(2) CPE may induce an endotoxin-like shock response (possibly due to CPE effects on immune cells), triggering cardiorespiratory failure(37).

(3) CPE may interfere with neuromuscular transmission by inducing increased exocytosis in neurosecretory cells(38, 39, 42). *In vivo*, these different mechanisms are likely to act synergistically, i.e., when CPE becomes systemically(40).

Other toxin :

Alpha toxin(α-toxin)

Alpha toxin is commonly procuced by all five types (A, B, C, D, E) and is a phospholipase C that can hydrolyze lecithin into phosphorylcholine and diglyceride and is believed to be a major factor responsible for the organism's tissue pathology (41). This is the predominant product of *C. perfringens* type A. Therefore, type A exhibits several powerful toxicities, and infection with type A may result in myonecrosis, hemolysis, and increase

in vascular permeability, and platelet aggregation(42). The major lethal effect associated with this toxin is gas gangrene in humans.

Beta toxin (β-toxin)

Beta toxin is a major lethal toxin produced by type B and C strains of *C. perfringens* and is a single-chain polypeptide of appoximately 40 kDa which is highly sensitive to trypsin(43). The beta toxin is known to play a major role in the pathogenesis of necrotic enteritis in humans and animals (3 (44). In human, the disease has been termed pig-bel which is caused by *C. perfringens* type C infection and which shows clinical signs of vomiting, abdominal pain, and bloody diarrhea(45).

Also, necrotic enteritic associated with toxingenic *C. perfringens* has been reported in calves, lambs, and piglets(46). In experimental infection and clinical studies(47), the presence of trypsin or protease inhibitors in the gut has been shown to be the most important cofactor in beta toxin-induced necrotic enteritis.

However, it remains unclear whether the presence of dietary trypsin inhibitors is a prerequisite for the disease. Also, type B of this organism was identified as a causative agent of enterotoxemia or necrotic enteritis in foals, lambs, sheep, and goats(46).

Epsilon toxin (Ɛ-toxin)

Epsilon toxin prodeced by types B and D of *C. perfringens* is responsible for a rapid fatal enterotoxemia in economically important livestock(47). This toxin is secreted as a relatively inactive protoxin and is activated to a potent heat-labile toxin with the loss of an N-terminal peptide by a proteolytic ensyme procuced by the organism(48). The activated protein is highly toxic and can have lethal, dermonecrotic, and edematic activities(49-51).

The effect on the brain is the most critical since the effect can induce death by cerebral edema and necrosis of brain tissue.

Iota toxin (I-toxin)

Iota toxin is produced only by type E of this bacterium and is also produced as a protoxin(52). The protoxin can permeate the vascular wall as a result of proteolytic activation(53). This toxin consists of two independent polypeptides : Ia, which an ADP transferase, and Ib, which is involved in the birding and internalization of this toxin into the cell. Although they can be distinguished immunologically and biochemically, their activities assist each other to produce toxicity such as dermonecrosis in mice(52). This toxin has also been implicated in calf and lamb enterotoxemia(54).

Clinical Significance

***C. perfringens* food poisoning**

C. perfringens generally ranks behind *Salmonella spp*. and *Staphylococcus aureus* as the third most common cause of food poinsoning in the United States

and elsewhere(3). Almost all U.S.A. outbreaks and causes of *C. perfringens* food-borne gastroenteritis appear to be due to type A strains(3, 55). In *C. perfringens* type A food-borne disease, the food vehicle is almost always an improperly cooked meat.,(e.g., beef, turkey, chicken, or pork) or a meat product, such as gravy, that has cooled slowly after cooking or may have been inadequately reheated(3, 56). Spores surviving the initial cooking germinate, and vegetative cells proliferate during slow cooking or insufficient reheating. *C. perfringens* type A food-borne illness should be suspected when there is an outbreak of diarrhea with crampy abdominal pain within about 7 to 15 h after the consumption of a suspected food(3, 55). However, the incubation period may range up to 30 h. Most patients are afebrile; nausea and vomiting occur in less than a third of patients, and the stools are frequently foamy and foul smelling. Illness results from the ingestion of food with about 10^8 viable vegetating cells, which in the alkaline environment of the small intestine undergo sporulation, producing an enterotoxin in the process, CPE, which accumulates intracellularly in sporulating *C. perfringens* cells, is released into the intestine at the completion of sporulation, as the sporulating cells lyse to release their endospore(57).

From the animal studies(30), the small intestine should be the site of action of the released CPE. After its release into the lumen of the small intestine, enterotoxin rapidly binds to the brush broder membranes of small intestinal epithelial cells, where it exerts its characteristic cytotoxic action. This action, in turn, is believed(26) to lead to the development of the diarrhea and cramping symtoms characteristic of *C. perfringens* type A food poisoning(25).

Because *C. perfringens* cells must complete sporulation before any CPE can be released into the intestine and this sporulation process typically takes 8 - 15 h, the first symtoms of *C. perfringens* type A food poisoning do not typically start until around 12 after ingestion of contaminated foods(28).

Somewhat contrary to its mild reputation, *C. perfringens* type A food poisoning ranks as the fifth leading cause of death from food poisoning in the U.S.A., with most deaths occurring in the elderly or in debilitated individuals(1, 4-6).

II. Detection of enterotoxigenic *C. perfringens*

Phenotypic methods

1. Immunodiffusion (double diffusion in two dimensions)

In this procedure antigen and antibody are allowed to migrate towards each other in a gel and a line of precipitation is formed where the two reactants meet. As this precipitate is soluble in excess antigen, a sharp line is produced at equivalence, its relative position being determined by the concentration of the antigen and antibody in the agar. The local concentration of each reactant depends not only on its absolute concentration in the well, but also on its molecular size and there fore, the rate at which it is able to diffuse through the gel. Multiple line of precipitation will be present if the antigen and antibody contain several molecular species. Consequently, this technique has the particular advantage that several antigens or antisera can be compared around a single well of antibody or antigen. Reidar and Charles (1975) investigated for enterotoxin production of 66 strains of *C. perfringens* by

immunodiffusion technique. This report found many of the strains sporulated poorly in DS medium or did not survive the heat shocking. Sporulation is necessary for enterotoxin synthesis but good sporulation can also be seen in enterotoxin-negative strains. However, low sporulation frequency may explain the failure to detect enterotoxin in cell extracts from many of the strains investigated in this study(60).

2. Immunoelectrophoretic analysis

In the study of antibody-antigen interaction solely by simple diffusion. It could be possible if there are only a few components in the system. However, if there are multiple antigen reacting with several antibodies, the precipitin lines become difficult to resolve and impossible to interpret. Increased resolution can be obtained by combining electrophoresis with immnodiffusion in gels, in the technique known as immunoelectrophoresis; It is a powerful analytical technique with great resolving power as it combines prior separation of antigens by electrophoresis with immunodiffusion againt an antiserum.(61)

However, γ-globulins are exceptional in their cathodic migration; most other proteins move to the anode. This property is used to advantage in counterimmunoelectrophoresis to cause antibody and antigen to migrate towards each other in gel and form lines of precipitation. The technique is similar to immunodiffusion but much faster as it is electrically driven, and more sensitive as all the antigen and antibody are driven towards each other. Takashi (1977)(62) have been detected enterotoxin in faeces after *C. perfringens*

food-poisoning by immunoelectrophoresis and counterimmunoelectrophoresis technique. This tests have approximately the same sensitivity (0.5-1.0 μg/ml), but in assaying crude faeces extracts the latter was unsatisfactory due to some loss of sensitivity.

3. Enzyme-linked immunosorbent assay (ELISA)

ELISA is immunometric assay almost invariably require solid-phase system. There can be in different ways, for example; (a) with antiboday on the solid phase to capture antigen, which is then detected by a second labelled antibody directed against another epitope on the antigen; or (b) for the detection of antibody by adsorption of antigen on the solid phase, followed by binding of the antibody to be determined, which, in turn, is detected against the Fc region.

Birkhead, et al (1988)(63) detected enterotoxin of *C. perfringens* by reverse passive latex agglutination in fecal specimens from six of six ill and from none of four well patients who had eaten turkey, suggesting that this organism had caused the outbreak. This investigation suggested that detection of fecal *C. perfringens* enterotoxin was a specific way to identify this organism as the causative agent in food-poisoning outbreaks. These investigators also detected enterotoxin of *C. perfringens* by ELISA in fecal specimens from none of four well patients who had eaten turkey, one of four give the positive result. The concluded that ELISA provided less specificity than of RPLA in the detection of enterotoxin producting *C. perfringens.*

Songer and Meer (1996)(64) used ELISA compared with a PCR assay and a digoxigenin labeled probe assay in the enterotoxin detection. They concluded that gene detection assay for *cpe* gene may be preferable to serologic assays for enterotoxin detection(65).

4. Agglutination test

Particulate antigens may be cross-linked by antibody to give visible agglutination in a manner analogous to the formation of precipitates with soluble antigens. The agglutination reaction has principally been exploited using red cells as the particles. Other particles such as latex and bentonite suspensions have also proved useful. Antisera may be compared semi-quantitatively by determining the end points of their respective titration curves. The sera are diluted until they no longer give a visible reaction with antigen by agglutination. This is a measure of the relative antigen-binding capacity of a serum and can only be used to compare antisera when they are tested at the same time and with the same antigen. Conversely, the concentration of an antigen in solution may be determined by the degree of inhibition of a standard, homologous agglutination system. Saito, M. (1990)(66) have studied enterotoxin production by *C. perfringens* isolates from feces of humans and from food and water in Japan by reverse passive latex agglutination, which anti-enterotoxin was coupled to polystyrene latex particles for use as a reagent in RPLA. He used this method because the amount of enterotoxin produced by the isolates were differed. When large amount of enterotoxin was produced, it could be detected by gel-diffusion test

but when small amount of enterotoxin was provided, it had to be tested by the RPLA. However, it is difficult to test many isolates by using of the avaiable tests because the enterotoxin production depends upon the sporulating medium, heat treatment, and so on and that the amount of enterotoxin produced varies for different strains. Thus, it is difficult to evaluate production of enterotoxin by *C. perfringens*(17, 60).

5. Western blotting

In this technique a sheet of nitrocellulose is placed against the surface of an SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis) protein fractionation gel and a current applied across the gel (at right angles to its face), thus causing the proteins to move out of the gel and onto the nitrocellulose where they bind firmly by non-covalent forces. Two variants of the basic apparatus are available to accomplish this electrophoretic transfer, the so-called wet and semi-dry blotters. In wet blotters the gel and transfer membrane are immersed in large volumes of buffer whereas semi-dry blotters use only filter paper pad moistened with buffer. Kokai-kun, et al (1994)(67) performed the comparative study between the western immunoblot and gene detection assay in the identification of potentially enterotoxingenic isolates of *C. perfringens.* In this study, western immunobloting (to detect enterotoxin production *in vitro*) was compared with PCR assay and digoxigenin-labeled probe assays (to detect all or part of the *cpe* gene) as a method for determining the enterotoxigenicity of *C. perfringens* isolates. The *cpe* gene detection assays yielded reliable results with DNA purified from vegetation *C. perfringens*

cultures, while western immunoblots required *in vitro* sporulation of *C. perfringens* isolates to detect enterotoxin production. This result indicates that *cpe* gene detection and serologic CPE assays do not necessarily yield similar conclusions about the enterotoxiginicity of a *C. perfringens* isolates. It may be preferable to use *cpe* gene detection assay for evaluation.
C. perfringens isolates enterotoxigenicity and thereby avoid potentical false-negative conclusion which may occur with serologic assays.

The demonstration of the enterotoxigenicity of *C. perfringens* with phenotypic methods has produced unreliable results because many strains of *C. perfringens* do not sporulate well of at all in the recommended sporulation media(59). Several sporulation media have been proposed but unfortunately there seems to be no universal medium that will encourage sporulation in all strains.

During recent years, potentially enterotoxigenic *C. perfringens* strains have been identified successfully with genotypic methods based on hybridization or polymerase chain reaction (PCR) amplification of the *cpe* gene(59).

Genotypic methods

Polymerase chain reaction (PCR)

PCR is an engymatic method of making multiple copies of a pre-selected segment of DNA. This amplification process is achieved with two synthetic oligodeoxynucleotide primers (A and B), a thermostable DNA polymerase, and the four deoxyribonucleotide triphosphates action on the

template DNA. The first primer (A) matches part of the Watson strand at one end of the segment, while the second primer (B) matches the Crick strand at the other end. When the template DNA is denatured by heating and then cooled, the primers anneal to their targets on the single-stranded templates. The DNA polymerase then extends the primers at their 3′-ends. Since the 3′-ends of the primers point toward each other, repeated cycles of heating and cooling lead to a chain reaction, an exponential synthesis of many copies of the specific segment bounded by the two primers. After twenty cycles of heating and cooling, one expects 2^{21} (i.e. more than 10^{6}) copies of the specific segment to be produced with a few hours will generate sufficient product ("amplicon") to be visualized and sized directly in an agarose or polyacrylamide gel and ethidium bromide staining(68).

This procedure has been applied to detection of enterotoxigenic *Vibrio cholerae* (69). The gene encoding *C. perfringens* enterotoxin has recently been sequenced (70). Later the researchers prepared extension primers from the base sequence of the enterotoxin gene to apply them to the detection of the enterotoxin gene by the PCR procedure, example Saito, et al (1992) detected enterotoxigenic *C. perfringens* isolates from food poisoning by PCR procedure, which prepared two primers from the DNA sequence of the gene encoding the enterotoxin and attempted the *in vitro* amplification of the gene by use of the two primers, the enterotoxin gene of enterotoxigenic *C. perfringens* was specifically detected and no such gene was detected in non-enterotoxigenic *C. perfringens*, showing the specificity of the procedure.

III. *C. perfringens* typing techniques

A convenient way of classifying typing systems is to divide them into phenotypic techniques, i.e., those that detect characteristics expressed by the microorganisms, and genotypic techniques, i.e., those that involve direct DNA-based analyses of chromosomal or extrachromosomal genetic elements.

Phenotypic techniques

Various methods have been recommended to use as the typing techniques. These included :

1. Biotyping

Biotyping makes use of the pattern of metabolic activities expressed by an isolates and may include specific biochemical reactions, colonial morphology, and environmental tolerances (e.g., the ability to grow on certain media or at extremes of pH or temperature). In general, however, biotyping has only limited ability to differentiate among strains within a species, and consequently, the technique had relatively poor discriminatory power. Hobbs et al (1953)(71) distinguished (the) food poisoning strains of *C. perfringens* from another *C. perfringens* by the production of heat resistant spore poisoning isolates are generally more heat resistant than spores made by *C. perfringens* isolates from other sources(5). However, it has currently mentioned that some isolates from food poisoning outbreaks in both U.S.A. and U.K. produced heat susceptible spores (13).

2. Antimicrobial susceptibility testing

Clinical microbiology laboratories routinely test most bacterial isolates for susceptibility to a panel of antimicrobial agents. Both manual and automated methods are widely available, rigorously quality controlled, typically easily performed, and relatively inexpensive. These data are readily available to clinicians and infection control practitioners. The identification of a new or unusual pattern of antibiotic resistance among isolates cultured from multiple patients is often the first indication of an outbreak. Antibiotic susceptibility testing has relatively limited utility not only because phenotypes vary but also because antibiotic resistance is under extraordinary selective pressure in contemporary hospitals. There are multiple genetic mechanisms by which a given strain can become abruptly resistant to a particular antibiotic. These include spontaneous point mutations and the acquisition of specific resistance genes via plasmids and transposons from other strains or even other species. Since a single plasmid or compound transposon can carry multiple resistance determinants, resistance to multiple antimicrobiol agents may be acquired simultaneously. On the other hand, in the absence of specific selective pressure, such elements may be lost(72). As a consequence of these various genetic mechanisms, different strains may develop similar resistance patterns and, conversely, the susceptibility patterns of sequential clinical isolates representing the same strain may differ for one or more antibiotic(73).

Eventhough the antibiotic susceptibility patterns appeared more discriminative than the genotypic methods, antibiotic susceptibility testing has

relatively limited utility in epidemiological studies because antibiotic resistance is affected by extraordinary selective pressure in contemporary hospitals(74).

Penicillin G shows excellent activity against most but not all strains of *C. perfringens* and has traditionally been considered the antibiotic of choice for the clostridia in general. However, resistance is increasing among *C. perfringens* to the extent that alternative antimicrobial agents may need to be considered(3).

3. Serotyping

Serologic typing (serotyping) is based on the long-standing observations that microorganisms of the same species can differ in the antigenic determinants expressed on the cell surface. Many different surface structures, including lipopolysaccharides, capsular polysaccharides, membrane proteins, and extracellular organelles (e.g., flagella and fimbriae) exhibit such antigenic variation.

The available rapid, reliable serologic assays for detecting antigen-antibody reactions use a wide variety of methods, including direct agglutination, latex coagglutination, enzyme labelling, and fluorescence labelling. If high-quality commercial reagents are not available, the preparation of specific typing antibodies is a difficult process and is generally restricted to reference and research laboratories. Serotyping often exhibit poor discriminatory power, even for bacterial species with large numbers of antigenic variants, because many strains may represent only a few serotypes or may be nontypeable(18).

Mahony, et al (1992) used to serotyping technique to indentify strains of *C. perfringens*. About 21% of the strains tested were non-typable with the range of available antisa(75).

4. Bacteriophage and bacteriocin typing

Among species for which numerous lytic bacteriophages have been identified, strains can be characterized by their patterns of resistance or susceptibility to a standard set of phages. Because of the need to maintain stocks of biologically active phages and control strains, phage typing is available only at reference laboratories. Even for experienced workers, the procedure is technically very demanding and suffers from considerable experimental as well as biologic variability. Since many strains are nontypable with the standard phages, additional, experimental phages often need to be considered.

In bacteriocin typing, and isolate is assessed for susceptibility to a set of bactericidal peptides produced by selected strains(76). Although the method has been useful for certain pathogens, such as *C. perfringens,*(77). bacteriocin typing has limitation similar to those described for phage typing.

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Table 1. Characteristics of phenotypic typing systems.

Typing system	Proportion of strains typeable	Reproducibility	Discriminatory power	Ease of interpretation	Ease of performance
Biotyping	All	Poor	Poor	Excellent	Excellent
Antimicrobial susceptibility	All	Fair	Poor	Excellent	Excellent
Serotyping	Most	Good	Fair	Good	Fair
Bacteriophage typing	Most	Fair	Fair	Fair	Poor

Typing methods that assess phenotypic differences are inherently limited by the capacity of microorganisms to alter the expression of the underlying genes(77). Such changes may occur unpredictably or in response to various environmental stimuli(78). In addition, point mutations representing a single nucleotide in the entire chromosome can result in the abnormal regulation or function of the gene responsible for a particular phenotypes. Thus, isolates that represent the same strain and are genetically indistinguishable (or almost so) can vary in the phenotype. Some phenotypic approaches, such as serotyping and bacteriophage typing, require specific reagents for detecting individual types. Since the available materials may not

be appropriate for all strains, a relatively large fraction of strains may give a null phenotype and consequently be nontypable. The summary of the characteristics of the phenotypic typing systems was shown in table 1.

Genotypic Techniques

Because problems with typeability, reproductibility, or discriminatory power been associated with many phenotypic techniques, numerous systems using DNA-based methods have been developed. Initially, these techniques were used only in a few research laboratories, but they have been rapidly becoming more widely available in clinical practice. In addition to the technical intricacies of these methods, the substantial complexities involved in effectively interpreting the results are becoming increasingly apparent. The methods based on genotypics techniques were as followed:

1. Restriction Endonuclease Analysis of Chromosomal DNA(REA)

A restriction endonuclease enzymatically cuts ("digest") DNA at a specific ("restricted") nucleotide recognition sequence. Both the recognition sequence of the enzyme and the composition of the DNA influence the number and sizes of the restriction fragment generated by digesting a given piece of DNA. Thus, an enzyme whose recognition sequence will generate fewer and larger restriction fragments than an enzyme recognizing sequences of only adenine and thymine. An enzyme that recognizes a sequence of 6 bp (a "6-bp cutter") typically has more recognition frequently, and consequently will generate more and smaller restriction fragments.

In conventional REA, bacterial DNA is digested with endonucleases that have relatively frequent restriction sites, thereby generating hundreds of fragments ranging from ~0.5 to 50 kb in length. Such fragments can be separated according to the size using agarose gel electrophoresis, The restricted fragment patterns can be detected by staining the gel with ethidium bromide and examining it under UV light. Different strains of the same bacterial species have different REA profiles because of variations in their DNA sequences that alter the number and distribution of restriction sites(79).

The major limitation of REA is the difficulty in comparing the complex profiles, which consist of hundreds of bands that may be unresolved and overlapping. In addition, restriction fragments derived from plasmids may confound the patterns. Thus, isolates that differ only in their plasmid contents may be considered different strains.

2. Southern Blot Analysis of RFLPs

REA patterns are complex because the ethidium bromide stain detects all of the hundreds of fragments generated by the restriction enzymes used. In contrast, Southern blot analyses detect only the particular restriction fragments associated with specific chromosomal loci. Southern blots, named after the investigator who first described the technique(80), are prepared by digesting bacterial DNA, separation the restriction fragments by agarose gel electrophoresis, and then transferring ("blotting") the fragment onto a nitrocellulose or nylon membrane. The fragment(s) containing specific sequences (loci) is the detected by using a labelled piece of homologous DNA

as a probe. Under the appropriate conditions, the probe binds ("hybridizes") by complementary base pair matching only to those fragments containing identical or nearly identical nucleotide sequences. Variations in the number and sizes of the fragments detected are referred to as restriction fragment length polymorphisms (RFLPs) and reflect variations in both the number of loci that are homologous to the probe and the location of restriction sites within or flanking those loci. All strains carrying loci homologous to the probe are typable, and the results are, in general, highly reproducible. The discriminatory power of an analysis is directly related to the frequency with which the fragments detected vary in number and/or in size. Thus, the choice of probes is a critical consideration.

Ribotyping refers to a southern blot analysis in which strains are characterized for the RFLPs associated with the ribosomal operon(s). Operons are clusters of genes that share related functions and are often coordinately regulated; the ribosomal operons comprise nucleotide sequences coding for 16 SrRNA, 25 SrRNA, and one or more tRNAs. Ribosomal sequences are highly conserved, and probes prepared from isolated *E.coli* rRNA or a cloned ribosomal operon (rrn)(81) hybridize to the chromosomal ribosomal operons of α wide range of bacterial species. All bacteria carry these operons and are therefore typeable. In general, ribotypes are stable and reproducible, with isolates from an outbreak typically having the same ribotype(82).

For organisms with multiple (five to seven) ribosomal operons, such as *E.coli* and *Klebsiella*, *Haemophilus,* and *Staphylococcus* spp., ribotype

patterns commonly have 10 to 15 bands and moderate discriminatory power. Nevertheless, epidemiological unrelated isolates not infrequently demonstrate the same pattern, limiting the utility of the mothod. For example, ~20% of 188 bloodstream isolates of *E.coli* from each of three geographically dispersed sites (Massachusetts, California, and Kenya) represented the same ribotype, although another genotypic technique (i.e., pulsed-field gel electrophoresis) generally resolved each isolate as a distinct strain. Isolates of this ribotype typically expressed adhesin and hemolysin virulence factor; as discussed above, virulent genotypes are frequently over represented among collections of bacterial pathogens. For bacterial species with only a single ribosomal operon, such as mycobacteria, ribosomal detects only one or two bands and has limited utility for epidemiological studies(83).

3. PFGE of Chromosomal DNA

As noted above, a major limitation of REA with enzymes that have ralatively frequent recognition sites is the difficulty of overlapping, poorly resolved restriction fragments. If the bacterial genome is digested with enzymes that have relatively few restriction sites, then considerably fewer but much larger restriction fragments are generated. Until relatively recently, the characterization of large DNA fragments was limited by two major factors. First DNA fragments of $\geq$ 25 kb are separated poorly or not at all by conventional agarose gel electrophoresis; second DNA prepared in solution is spontaneously sheared into random fragments typically of $\leq$ 100 kb. (84-88) PFGE, developed by Schwartz and Cantor in 1984(89), is a variation of

agarose gel electrophoresis in which the orientation of the electric field across the gel is charged periodically ("pulsed") rather than being kept constant as in the conventional agarose gel electrophoresis used for the REA and Southern blot studies described above. For technical reason(90), this critical modification enables even megabaseize DNA fragments to be effectively separated by size.

Suitable unshared DNA is obtained by embedding intact organisms in agarose plugs ("inserts") and then enzymatically lysis the cell wall and digesting the caller proteins. The isolated genomes are then digested *in situ* with restriction enzymes that have few recognition sites(91). PFGE analysis of such digests provides a chromosomal restriction profile typically composed of 5 to 20 distict, well-resolved fragments ranging from approximately 10 to 800 kb. All bacterial isolates are theoretically typeable by PFGE, and the results are highly reproducible. The relative simplicity of the restriction profiles greatly facilitates the analysis and comparison of multiple isolates.

Total DNA profiles obtained by PFGE method gave excellent typability, as all strains yielded a DNA profile and presented very good stability. Reproducibility was very good in triplicate experiments, and comparison of different gels was facilitated by including a reference strain. The discriminatory power also appear good(104). Ridell et al used the pulsed-field gel electrophoresis in the epidemiologic study on of *C. perfringens* isolated from food and diarrheal feces in associated with 36 separate food-poisoning cases or outbreaks in Germany. The results with PFGE showed that this is a frequent phenomenon. Whether this really means that the etiology of

C. perfringens food poisoning is frequently multiclonal. The existence of multiple *cpe*-positive clones in a common outbreak must be taken into consideration when applying typing methods to trace the veticle of the food poisoning(6). However, PFGE has two notable limitations. First, because of the need for all buffers and enzymes to be diffused into the agarose insert, the preparation of suitable DNA involves several extended incubations and takes from 2 to 4 days. This effort is partially offset by the fact that DNA in agarose is stable for years at $4^{O}C$ and can easily be released into solution for use in other protocols. Second, PFGE requires relatively expensive, specialized equipment.

The conclusion of the characteristics of genotypic typing systems was show in table 2.

Table 2 Characteristic of genotypic typing sytems.

Typing system	Proportion of strains typeable	Reproducibility	Discriminatory power	Ease of interpretation	Ease of performance
REA	All	Good	Good	Poor	Excellent
Ribotyping	All	Excellent	Fair	Good	Poor
PFGE	All	Excellent	Excellent	Excellent	Good

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IV. *Clostridium perfringens* type A food poisoning outbreaks.

Incidence

C. perfringens type A food poisoning annually ranks among the most common food-borne diseases in the United States and Europe. The Centers for Disease Control and Prevention (CDC) reported that there were 190 outbreaks (10.2% of total bacterial food-borne disease outbreaks) of *C. perfringens* type A food poisoning in the United States, from 1973 to 1987 involving 12,234 cases (11.2% of total cases of bacterial food-borne diseases) and 12 deaths (~5% of total deaths from bacterial food-borne diseases). However, since most cases of this disease go unreported, these official statistics significantly understate the true prevalence and impact of *C. perfringens* type A food poisoning. Todd had estimated that there were actually 652,000 cases of *C. perfringens* type A food poisoning in the United States each year, with an average of 7.6 deaths per year and annual costs of $123 million (93, 94).

C. perfringens type A food poisoning can occur at any time of year but is slightly more common during summer months(56), perhaps because higher ambient temperatures facilitate temperature abuse of foods during cooling and holding(95).

Food vehicles

In the 1973-1987 CDC reports, meat and poultry continued their traditional roles as the most common food vehicles for *C. perfringens* type A food poisoning in the United States. Beef accounted for nearly 30% of all

C. perfringens type A food poisoning outbreaks during this period, with turkey and chicken together accounting for another ~15%. The CDC statistics also indicated that Mexican foods containing meats were emerging as an other important vehicle for *C. perfringens* type A food poisoning(5).

Contributing factors

C. perfringens type A food poisoning almost always results from temperature abuse during the cooking, cooling, or holing of foods. The CDC reports (96) that improper storage of holding temperatures contributed to 97% of all recent *C. perfringens* type A food poisoning outbreaks, while improper cooking was a factor in 65% of these outbreaks. Other major contributing factors for *C. perfringens* type A food poisoning include contaminated equipment and poor personal hygiene, which were involved in 28 snd 26% respectively, of recent outbreaks of this food poisoning. As introduced earlier, the importance of temperature abuse in *C. perfringens* type A food poisoning is not surprising given the relative heat tolerance of *C. perfringens* vegetative cells and, more particularly, the high heat tolerance of *C. perfringens* spores. As also mentioned previously, incomplete cooking actually promotes this illness by increasing the germination rate of *C. perfringens* spores present in foods which were after outgrowth of these spores into new vegetative-abused foods that are cooled or stored improperly(97).

Examples of Recent Outbreaks

Since consideration of recent outbreaks often illustrates important concepts underlying bacterial food poisoning, a few recent reports describing *C. perfringens* type A food poisoning outbreaks in the United States and the United Kingdom will be discussed briefly.

In 1994, the CDC published a report on an investigation of two outbreaks of *C. perfringens* type A food poisoning that were associated with St. Patrick's Day meals(98). The first of these outbreaks occurred in Cleveland, Ohio, and involved 156 persons, all of whom acquired *C. perfringens* type A food poisoning from ingesting corned beef that had been prepared at a local delicatessen. During its preparation, the corned beef had been boiled for 3 h and then allowed to cool slowly (at room temperature) before refrigeration. Four days later, portions of this corned beef were warmed to 18.8°C and served; some sandwiches prepared with this corned beef were held at room temperature from the time of their preparation (at 11:00 a.m.) until they were eaten throughout the afternoon.

The second St. Patrick's Day outbreak described in the CDC report occurred in Virginia and involved 86 persons who attended a traditional St. Patrick's Day dinner. This dinner also included corned beef, which was later found to contain large numbers of *C. perfringens* cells. The corned beef involved in the Virginia outbreak was a frozen, commercially prepared, brined product that had been thawed, cooked in large (10-lb [ca. 4.5-kb]) pieces, stored in a refrigerator, and held for 90 min under a heat lamp before being served.

These two outbreaks illustrate the typical association between *C. perfringens* type A food poisoning and meat (particularly beef) vehicles and the importance of temperature abuse as a contributing factor to this foodborne disease. Specifically, in the Ohio delicatessen outbreak, the corned beef was clearly cooled too slowly after cooking and then was not reheated at sufficiently high temperatures before being served. In the Virginia outbreak, the beef was first cooked in excessively large portions and then not adequately reheated before being served. Interestingly, the Virginia outbreak is somewhat unusual in that it involved a commercially prepared, brined meat product.

Another recent report described a *C. perfringens* type A food poisoning outbreak that occurred in a British hospital and sickened 17 patients (99). Epidemiologic investigation of this outbreak found large number of *C. perfringens* cells present both in the stools from ill patients and in vacuum-packed pork served to the patients, implication this pork as the presumed food vehicle for the outbreak. The pork had apparently become contaminated with large numbers of *C. perfringens* as a result of very slow cooling after cooking at a commercial meat preparation facility. This British outbreak appears typical for a *C. perfringens* type A food poisoning outbreak with respect to the implicated food vehicle (meat), epidemiology (like the Virginia St. Patrick's Day outbreak mentioned above) the British hospital outbreak involved a large number of cases in an institutional setting), and cause (temperature abuse). However, this British outbreak, like the Virginia outbreak, is somewhat unusual in that it involved a commercially prepared food product. Perhaps the

involvement of vacuum-packed meat in the British outbreak presages future problems with increasingly popular, vacuum-packed, precooked meat products serving as common vehicles for *C. perfringens* type A food poisoning. Vacuum-packed foods would appear to provide an ideal anaerobic environment for growth of clostridia, including *C. perfringens.*

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CHAPTER IV

MATERIALS AND METHODS

1. Chemical and reagents

Most of the chemical agents used in this study were molecular biological grade. Name lists of all chemical reagents and instruments were shown in the appendix.

2. *C. perfringens* isolates

2.1 From food and water samples.

80 *C. perfringens* isolates from 700 water and food samples collected from various sources all over the country between 1995-1999 were kindly provided from Food Division, Department of Public Health. Details of the samples were show were shown in table 3.

Table 3. Food and water sources.

Sources of Samples	**No. of isolates**
Fresh food	30
Preserve food	9
Dried food	2
Water & beverage : drink water, ice water, soft drink, mineral water, coconut milk, iced black coffee.	39
Total	80

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2.2. From food poisoning outbreaks in Japan

5 isolates from patients, with food poisoning outbreak were kindly provided from Institute of Anaerobic Bacteriology, Gifu, Japan. The isolates were designated as WA, WB, WD, W35, W40, respectively.

2.3. Reference strains

C. perfringens NCTC 8239 was used as control for toxin positive strain and *C. perfringens* ATCC 14367 was used as control for toxin negative strain.

3. Stool samples

80 stool samples from admitted-patients with diarrhea during their hospital stay at Siriraj Hospital and 1 stool sample from non admitted-patient with acute diarrhea were obtained during the 6 months of the specimens collection period.

4. Identification of *C. perfringens*

Stool samples were cultured and identified according to the method reccommened by Wadworth (88). Brieefly, stool samples were streaked on sheep blood agar plate and incubated anaerobically at 37°C for 48 hours. The colonies after 48 hours incubation were examined for the specific characteristics including double hemolysis zone on sheep blood agar. The colony was tested for anaerobes by streaking on two sheep blood agar plates one was incubated anaerobically and another was incubated aerobically at

$37^{o}C$ for 48 hours. *C. perfringens* colonies appeared only on the anaerobic condition but not on the aerobic one.

5. Confirmed identification of all *C. perfringens*

5.1 Colony morphology

The colonies after 48 hours incubation were examined for their specific characteristics including double hemolysis zone on sheep blood agar, gray color, circular on the sheep blood agar.

5.2 Catalase test (slide method)

A well isolated colony was transferred with a flame-sterilized looped and inoculated to the surface of a clean glass slide. A drops of 3% H_2O_2 was added to the cells. Bubble of oxygens was seen if the isolates was catalase positive.

5.3 Gram staining procedure

The organisms were smeared on a clean slide and allowed to dry. The slide was gentlely heated with a flame to fix the smear. Gram crystal violet was dropped on the smear. After 1 minute, the slide was then washed with water and drained. Next, gram iodine solution was dropped on the smear, and washed with water for 1 minute. The smear was decolorized with ethanol (95%) and then washed with water. Five percent safranin solution was dropped on the smear for 30 second. The smear was washed with water and then allowed to dry. *C. perfringens* is gram positive rod

5.4 Nagler's test

Prior to inoculation, an egg yolk agar plate was laid with *C. perfringens* antitoxin strip (filter paper impregnated with antitoxin). The plate was allowed to dry. The inoculum was streaked starting on the section without antitoxin of plate and the end at the strip. The plate was incubated for 48 hours. The Nagler's positive was shown by the inhibition of the lecithinase enzyme produced by the isolate in the area of antitoxin diffusion.

5.5 Lactose fermentation

Litmus milk was inoculated with the organism and incubated at 37°C under anaerobic condition for 2 days. Rapid fermentation of lactose could occur with the subsequent development of a characteristic "stormy clot" reaction.

5.6 Indole production test

Trypicase nitrate broth was inoculated with the organism and incubated at 37°C in anaerobic condition for 2 days. The presence of indole was tested using the Kovac's method, 0.3 ml of Kovac's solution was added into 6 ml of broth culture.The alcohol layer will separate from the aqueous layer upon standing, and a reddening of the alcohol layer within a few minutes indicates that indole is presented.

5.7 Identification by RAPID ID 32 A identification kit (Biomerieux).

The identification process was done according to the company's guideline. Briefly as followed.

1. Preparation of the inocolum
    1.1 Using a sterile swab, harvest the 24 hour growth obtained on the blood agar plate and inoculate in 3 ml suspension medium (or sterilie distilled water).
    1.2 Make a suspension wath turbidity equivalent to standard Mcfarland solution No.4.
2. Inoculation bacterial suspension
    2.1 Homogenize the inoculated ampule of suspension medium and inoculate the strip by distribute 55 ul of the suspension into each cupule with the ATB Electronic Pipette.
    2.2 Add 2 drops of mineral oil to the URE cupule. Place the lid on the strip and incubate at 37°C for 4 hours in aerobic conditions.
3. Preparation of the strip
    3.1 Remove the strip from its packing and place it at room temperature.
    3.2 Record the strain reference on the elongated flap of the strip.
4. Reading the strip
    4.1 Testing reagent including nitrate, indole were added according to the kit instruction.

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4.2 Read after 5 minutes by using the mini API instruments. The reader records the color of each cupule and transmits the information to the computer.

5. Interpretation

Identification could be made after automatic reading according to the biochemical characteristic of *C. perfringens* in table 4. The results transmitted to the computer were interpreted by the indentification software.

All *C. perfringens* isolates were maintained in 15% glycerol broth (anaerobic stock cultures) at -20°C

Table 4 Biochemical characteristics of *C. perfringens*

Test	**Result**
URE-UREase	-
ADH-Arginine DiHydrolase	+
αGAL- alpha GALactosidase	+
βGAL- GALactosidase	+
βGP- beta Galactosidase 6 Phosphate	-
αGLUb-alpha GLUcosidase	+
βGLU-beta GLUcosidase	-
αARA-alpha ARAbinosidase	-
βGUR-beta ClucURonidase	-
βNAG-beta N-Acetyl-Clucosaminidase	+

Table 4 (Continue)

MNE-MaNnosE fermentation	+
RAF-RaFfinose fermentation	-
GDC-Glutamic ac. DeCarboxylase	+
αFUC-alpha FUCosidase	-
NIT-Reduction of NIT rates	+
IND- INDole Production	-
PLA-Phosphatase Alcaline	+
ArgA-Arginine Arylamidase	-
ProA-Proline Arylamidase	-
LGA-Leucyl Glycine Arylamidase	-
PheA-Phenylalanine Arylamidase	-
LeuA-Leucine Arylamidase	-
PyrA-Pyroglutamic ac. Arylamidase	-
TyrA-Tyrosine Arylamidase	-
AlaA-Alanine Arylamidase	-
GlyA-Glycine Arylamidase	-
HisA-Histidine Arylamidase	-
GGA-Glutamyl Glutamic ac. Arylamidase	-
SerA-Serine Arylamidase	-

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6. Detection of heat resistant spore.

The method was done according to the method by Hobbs, et al, (1969)(100). Briefly as followed:

6.1 The isolated organism was cultured in cooked meat medium at 37°C for 18 to 20 hours. The vegetative cell was then inactivated by heating at 75°C for 20 minutes, 0.8 ml of bacterial culture in cooked meat medium was transferred to 18 ml of DS medium (Duncan and Strong) for the promotion of sporulation. The culture was then incubated at 37°C for 24 hours.

6.2 The spore staining was performed by

- Smear the DS culture medium on glass slide
- Place the slide over a beaker of boiling water resting it on the rim with the bacterial film uppermost.
- When, within several seconds, large droplets have condensed on the underside of the slide, flood the smear with 5% aqueous solution of malachite green and leave to act for 1 min while the water continues to boil.
- Wash the smear with cold water.
- Treat with 0.5% safranine for 30 sec.
- Wash and dry and checked spores by microscope.

6.3 Heat resistant spore screening by:

- Inoculate one ml of the spore-forming *C. perfringens* culture into a tube containing 18 ml of cooked meat.
- Broth was heated at 100°C for 30 min.

- Steak the broth culture on sheep blood agar and add one ml of the broth into BHI broth.
- Incubated anaerobically for 24 hours.
- The growth culture indicated the presence of heat resistant spores of *C. perfringens*.

7. *C. perfringens* enterotoxin detection by reverse passive latex agglutination (RPLA) (Commercial kit : Oxoid). Briefly as followed :

7.1 Culture the isolated organism in cooked meat medium (oxoid) at 37°C for 18 to 20 hours, the inactivate vegetative cell by heating at 75°C for 20 minutes. Subculture to DS medium (Oxoid).

7.2 Inoculate 16 to 18 ml of the DS medium with 0.8 ml of the cooked meat medium culture and incubate at 37°C for 24 hours. Later, stain spore with malachite green and check spores by microscope.

7.3 After incubation, centrifuge at 900g for 20 minutes at 4°C and use the supernatant as the sample.

7.4 Arrange the 96-well plate so that each row consists of 8 wells. Each sample needs the use of 2 such rows.

7.5 Using a pipette, dispense 25 µl of diluent in each well of the 2 rows except for the first well in each row.

7.6 Add 25 µl of teat sample to the first and second well of each row.

7.7 Using a pipette and starting at the second well of each row, pick up 25 µl and perform doubling dilutions along each of the 2 rows. Stop at the 7th well to leave the last well containing diluent only.

7.8 Add 25 µl of sensitised latex on each well of the first row.

7.9 Add 25 µl of latex control to each well of the second row.

7.10 To mix the contents of each well, rotate the plate by hand.

7.11 Place the plate in a moisture box leave the plate undisturbed on a vibration-free surface at room temperature for 20 to 24 hours.

7.12 Examine each well in each row for agglutination against a black background indicating enterotoxin positive from each culture.

8. **Enterotoxin gene (*cpe* gene) detection by polymerase chain reaction (PCR) (Kato, et al 1991).** The method was as followed :

8.1 Preparation of DNA template

Brain heart infusion agar was streaked with the organism and incubated at 37°C under anaerobic condition for 24 hour. A single colony from plate was taken into a 1.5 ml microtube tube and centrifuge at 14,000 rpm for 30 sec. The supernatant was discarded and suspended pellet in 500 µl of lysis buffer # 1 (5 mg/ml lysozyme, 25% sucrose, 50 mMTris 5 mMEDTA, 50 mMNaCl). Incubated at 37°C for 15 minutes or more (DNA was still in cells at this step). Centrifuge at 14,000 rpm for 2 minutes. The supernatant was discarded and suspended pellet in 200 µl of lysis buffer # 2 (16µl of 10% sarkosyl, 4 µl of proteinase k; 5 mg/ml in distilled water, 180 µl of TEN; 10 mMTris, 1 mMEDTA, 10 mMNaCl). Incubated at 56°C for 60 minutes. Centrifuge at 14,000 rpm for 2 min to remove cells dibris and the supernatant was used as template DNA in the PCR.

8.2 Preparation of Reagent

- 10 mM Deoxynucleotide triphosphates (dNTPs)
- 10X $MgCl_2$ free buffer
- 25 mM $MgCl_2$
- 5 U/µl Tag DNA polymerase
- Primers for detection of target genes.

Target gene		5′-sequence-3′	Size of PCR
Enterotoxin	Ent 1	ATGTAATAGATAAAGGAGATGGTT	163
(Daube, 1994)	Ent 2	ATAAATTCAGAAGTAAATCCAACT	
enterotoxin	Ent 3	TTACCCAAGCTTTAATTCCTTCAGC	250
(Kim, unpublished)	Ent 4	CTTTAGCATTTTCGAACACCATTG G	

8.3 Preparation of PCR mixture

- PCR mixture of enterotoxin primer (ent_1, ent_2) solution

10X $MgCl_2$ free buffer (1X)

10mM Mixed dNTP (0.4 mM)

25 mM $MgCl_2$ (2.0 mM)

Distilled water

Each of ent_1 and ent_2 primer(0.4 µm)

5 U/µl Tag DNA polymerase (1.5 U/µl)

Template DNA (0.5 µl)

- PCR mixture for enterotoxin primer (ent_3, ent_4) solution

  10X $MgCl_2$ free buffer (1X)

  10mM Mixed dNTP (0.4 mM)

  25 mM $MgCl_2$ (2.0 mM)

  Distilled water

  Each of ent_3 and ent_4 primer (0.4 μm)

  5 U/μl Tag DNA polymerase (1.5 U/μl)

  Template DNA (0.5 μl)

8.4 Gene amplification procedure

Temperature and time profiles for amplification.

- Denaturation	94°C, 1.5 minutes
- Annealing	50°C, 1.0 minutes
- Extension	72°C, 1.0 minutes
Cycle	34 rounds

Ten μl of PCR product were then analysed by electrophoresis in 5% polyacrylamide gel.

8.5 Electrophoresis

The glass plates for pouring the gel was prepared. Five percent was polyacrylamide gel by combining 14.6 ml of distilled water, 3.3 ml of 30% acrylamide/ bisacrylamide, 2 ml of 5xTBE and 450 μl of ammonium persulfate. The gel solution was mixed, and poured. The appropriate comb was inserted immediately, The acrylamide was allowed to polymerize at room temperature for 45 minutes. When polymerization was complete, the comb

was removed and immediately rinsed out of the wells with water. The electrical tape was removed from the bottom of the gel. The gel was attached to the electrophoresis tank, which had already been filled with 1 X electrophoresis buffer (1XTBE, 89 mM Tris, 89 mM Boric acid, 2 mM EDTA, pH 8). Any air bubbles trapped beneath the bottom of the gel was removed with a bent pasture pipette . The DNA samples were prepared by mixing 10 μl of amplified product with 2 μl of 6X loading dye (0.25% Bromophenol blue, 0.25% Xylene cyanol FF, 0.4% Orange G, 10% ficall®400, 10 mM Tris-HCL (pH 7.5), 50 mM EDTA). Five μl DNA marker (1,353-bp ladder, which supplied the 6X loading dye) was also included and loaded into a slot of 0.5 cm long and 0.2 cm wide. The electrodes was connected to a power supply, pack polyacrylamide gel was run at 125 constant volt, time 30 minutes or until the marker dyes had migrated approach the edge of the gel. At the end of run, the plates was detached from the tank and the tape was removed from the sides. The gel was laid and the two glass plates were flatted on the bench. A corner of the upper glass plate was lefted up and pulled smoothly away, while the gel was still attached to the lower plate. The spacers was removed.The gel and its attached glass plate was submerged in the staining solution (0.5μl /ml of ethidium bromide in 1X TBE buffer) for 30 minutes and then destained for 15 minute with the deionized water. The stainned gel was photographed under UV illumination. The locasion of DNA band of interest was observed.

9. Alpha toxin gene (α-gene) detection by PCR

The method was performed according to the method by Yoo (1997). Briefly as followed.

9.1 The same DNA samples as in 6.1 were used to detect α-toxin.

9.2 Preparation of reagent

- 10 mM Deoxynucleotide triphosphates (dNTPs)
- 10X $MgCl_2$ free buffer
- 25 mM $MgCl_2$
- 5 U/μl Tag DNA polymerase
- Primers for detection target genes.

Target gene		5′-sequence-3′	Size of PCR
Alpha-toxin	α_1	GTTGATAGCGCAGGACATGTTA	402
(Yoo, H.1997)	α_2	AG CATGTAGTCATCTGTTCCAGCATC	

9.3 Preparation Of PCR mixture

PCR mixture for alpha-toxin primer (α1, α2) solution

-10X $MgCl_2$ free buffer (1X)

-10 mM Mixed dNTP (0.4 mM)

-25 mM $MgCl_2$ (2.0 mM)

-Distill water

-Each of α_1 and $\alpha_2$primer (0.4 μM)

-5 U/μl Tag DNA polymerase (1.5 U/μl)

-Template DNA (0.5μl)

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9.4 Gene amplification procedure

The same as in 6.4

9.5 Electrophoresis

The same as in 6.5

10. Preparation and separation of restriction fragments length polymorphism of chromosomal DNA by pulsed-field gel electrophoresis (PFGE)

Restriction fragments of chromosomal DNA of all *C. perfringens* isolates were prepared and separated by PFGE according to the method by Wongwanich(110). The method was as followed :

10.1 Sample Preparation

A single well-isolated colony on sheep blood agar was inoculated into 10 ml brain heart infusion broth (BHI broth) and incubated anaerobically at 37°C overnight. Ten millilitre of overnight culture was dispensed into each 10 ml snap-top tube and centrifuged at 3,800 rpm for 10 min at 4°C. Supernate was removed. Five ml of TES buffer was added, mixed with vortex, centrifuged at 3,800 rpm for 10 min at 4°C. TES buffer was used to adjust the turbidity with McFarland no.3. Low melting point agarose (Difco) in TES buffer was prepared in a flask to make the final concentration of 1.6% W/V then heated in the microwave for 30 sec. For each strain preparation, 0.5 ml of agarose was dispened into 5 ml snap-top tube which was kept in 50°C water bath, 0.5 ml of bacterial cell suspension was added into this tube. The mixture was dispensed into each well of the cold plug mold, and placed at 4°C for 20 min to solidfy. The cold plug mold

was prepared prior to the dispersion of the bacteria-agarose mixture by placing the mold in metal tray which was filled with ice and placed the tray in the refrigerator for at least 30 minutes.

Two ml of lysis buffer (10 mM Tris-HCL pH 7.6, 10 mM EDTA, 1 mg/ml mutanolysin) were dispensed into each labeled 15-ml round-bottom tube. For each strain, the plug was pushed out into the tube containing the lysis buffer and incubated overnight at 37°C shake in water bath. After that, the plug was added into the tube containing 3 ml of lysis solution I (1% SDS, 0.5 M EDTA, 20 mg/ml Rnase) and incubated overnight at 37°C in shaking water bath. After that, the plug was added into the tube containing 3 ml of lysis solution II (1\5 sarcosyl, 0.5 M EDTA, 20 mg/ml protease) and incubated overnight at 37°C. Finally the plug was added into the tube containing the PMSF solution (PMSF : 1XTE = 1:10) 3 ml and incubated in shaking water bath at 37°C for 1 hour. This step was repeated two times. The plug was washed with 0.5XTE 5 mM Tris, 0.5 mM EDTA, pH8) two times. At this step, the plug was ready for the restriction enzyme digestion.

10.2 Restriction enzyme digestion

The plug was placed on the sterile plastic plate. The 150 μl of 1xREB buffer was dropped on the plug and incubated at room temperature for 30 min. The plug was then cut into half which was then added into the microcentrifuge tube containing 1xREB buffer plus *Sma* I. The tube was mixed gently, and incubated overnight at 25°C. Another half plug was in 0.5 x TE at 4°C.

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10.3 Gel preparation, loading and running

The running gel was prepared using, the high-melting temperature agarose (Gibco BRL) by dissolving the agarose in 0.5xTBE to make the final concentration of 1% W/V. Plug of each isolate and lambda ladder marker in agarose plug DNA size standard was cut into the size of approximately 1 mm thick off the end of it by using a glass coverslip. All the plugs were loaded into a well of the gel. All the wells were then filled with 1% agarose low-melting temperature. The contour-clamped homogeneous field (CHEF) system (Bio- rad, US) was used. The gel was placed on a PFGE box which was already filled with 800 ml of 0.5xTBE. It was then electrophoresis at 200 V (6 v/cm), initial a time of 5s a final a time of 50s, run time for 24 hours. The remainder of the plug was stored at 4°C in microcentrifug tube containing 0.5XTBE.

10.4 Gel visualization

The gel was stained for 45 min with ethidium bromide solution (2µg /ml in water). It was destained with the deionized water for at least 15 min, and then photographed under UV illumination. According to Tenover ,et al (45) the patterns which were different from each other more than 3 bands were classified as different pulsotypes while the patterns which differed from each other less than 3 bands were classified as subtypes.

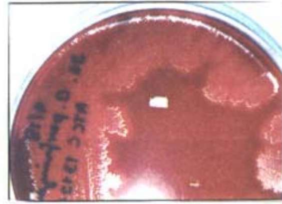

Figure 1A

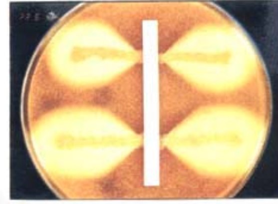

Figure 1B

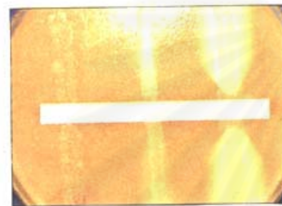

Figure 1C

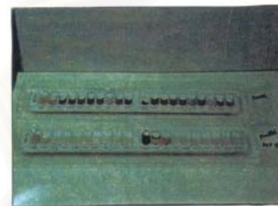

Figure 1D

Figure 1A	Colony of *C. perfringens*
Figure 1B	Nagler test (positive) of *C. perfringens*
Figure 1C	Nagler test (negative and positive) of *C. perfringens*
Figure 1D	Biochemical test for identification of *C. perfringens* (RAPID ID 32A)

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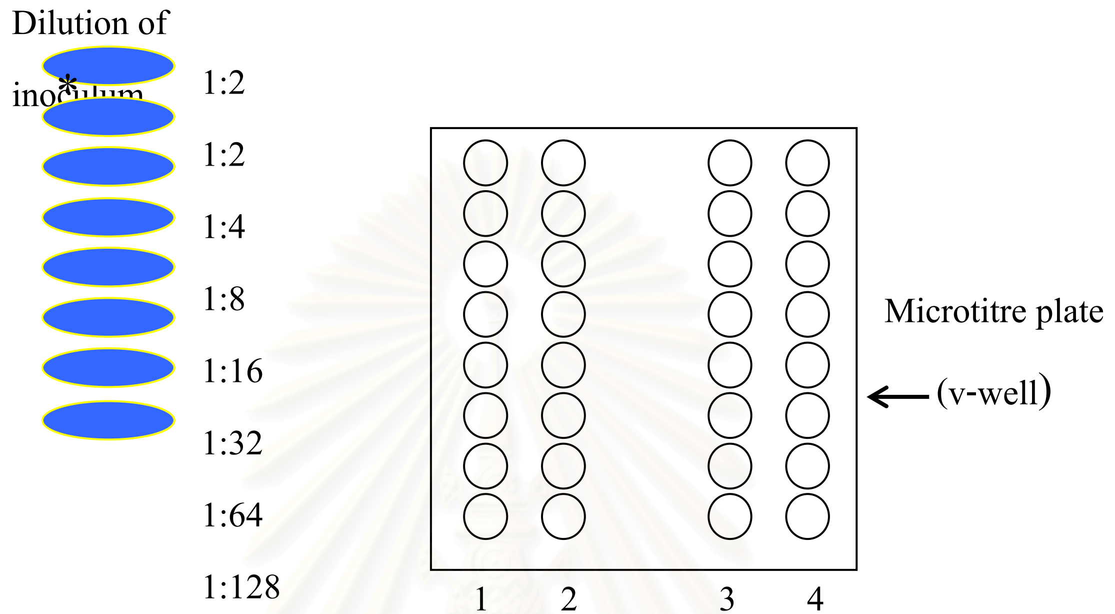
Reverse passive latex agglutination test (RPLA kit)



Column 1 = 2 fold dilution of organisms + latex (anti-CPE)

Column 2 = 2 fold dilution of organisms + latex (non anti-CPE)

Column 3 = 2 fold dilution known enterotoxin + latex (anti-CPE)

Column 4 = 2 fold dilution known enterotoxin + latex (non anti-CPE)

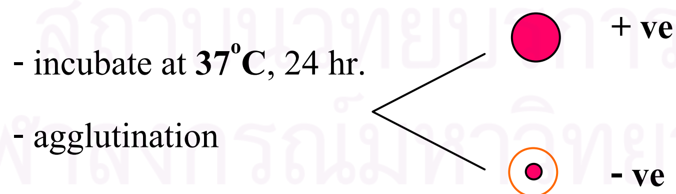


Fig 2 Diagramatic of RPLA kit test

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Figure 3 Electrophoresis System

Figure 4 Gel visualization

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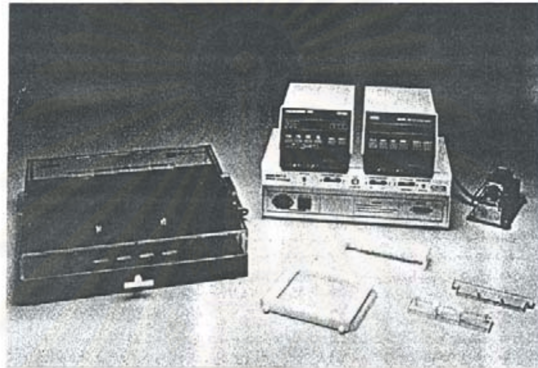

Figure 5 CHEF-DR II pulsed-field

Electrophoresis system

Figure 5 CHEF-DR II pulsed-field

Electrophoresis system

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Results

C. perfringens isolates from stool

As shown in Table5, a low number of *C. perfringens* were isolate from stool of the-patients with acute diarrhea at Siriraj Hospital. Only 20 out of 80 patients with diarrhea had the organism in the stools. During the 6 months of the stool specimens collection, there was only one stool specimen obtained from a non-admitted patient with acute diarrhea. And *C. perfringens* also was isolated from the stool.

Sporulation in Ds medium of *C. perfringens* from different soures

The sporulation *C. perfringens* from different sources was shown in table 6. Among all eighty nine *C. perfringens* isolates from food and water, 67 strains were able to sporulate in the medium at 37 $^{\circ}$C while only 9 out of the 20 isolates from stool of the admitted-patients with diarrhea were able to sporulate at 37 $^{\circ}$C. One isolate from non-admitted patient also sporulated at this temperature. Three out of 5 food poisoning isolates from Japan were able to sporulate included both positive control strain (NCTC 8239) and negative control strain (ATCC 14367).

When the temperature was increased to 43 $^{\circ}$C in order to stimulate more sporulation, similar result was obtained as when the temperature was 37 $^{\circ}$C.

Heat resistant spore of *C. perfringens* isolated from the different sources.

Table 7 showed the number of isolates producing heat resistant spore. None of the isolates from food, water, and stool of the admitted-patients as well as 2 out of 5 isolates from food-poisoning outbreak in Japan and negative control strain had heat resistant spore. The isolate from the non-admitted patient, 3 isolates from the food poisoning outbreak in Japan and the positive control strain (NCTC 8239) produced heat resistant spores (survived for 20 min at 100 $^{\circ}$C).

Enterotoxin detection by reverse passive latex agglutination (RPLA Kit)

Table 8 demonstrated the number of isolates with positive toxin detected by RPLA as compared to the total number of sporulating isolates. Only one isolate from stool of the non-admitted patient as well as the 3 food poisoning isolates from Japan produced enterotoxin. Positive control strain (NCTC 8239) produced enterotoxin while negative control strain (ATCC 14367) did not.

Detection of α-toxin gene by PCR

DNA was isolated from all *C. perfringens* and confirmed for the success in obtaining DNA by the PCR detection of α-toxin gene which should be presented in all isolates. The result obtained clearly shown that all isolates

were α-toxin gene positive indicating that *C. perfringens* DNA were correctly isolated from all isolates as shown in table 9. And showed in figure.

Detection of enterotoxin gene (*cpe* gene) by PCR.

Enterotoxin gene detection by PCR was performed using 2 different pairs of primers, (ent1, 2) and (ent3, 4). In table 10 similars results were obtained by using either pairs of primers. All isolates from food and water and from stool of the admitted-patients was found to be *cpe*-negative. Only the isolate from the stool of non-admitted patient and all 5 isolates from food poisoning outbreak in Japan (WA, WB, WD, W35, W40) were *cpe*-positive as well as the positive control strain (NCTC 8239). The negative control strain (ATCC 14367) was found to be *cpe*-negative.

Pulsotyes of *C. perfringens* isolated from different sources.

All 106 *C. perfringens* isolates were discriminated into 88 different pulsotypes according to the restricted chromosomal DNA fragment patterns from PFGE as shown in Fig 10-12 and table 11. There were 36 pulsotypes among the 40 isolates from food, 32 different types from 39 isolates from water, 16 different types from 21 isolates from stool, 4 different types from 5 isolates from food poisoning. Diagramatic patterns of all 88 pulsotypes were shown in Fig 13.

Similar pulsotypes among the different *C. perfringens* isolates.

Table 12 showed similar pulsotypes from different sources. Almost all *C. perfringens* isolates were individual distinct pulsotypes. There were not more than 2 isolates from the same or different sources which were in the same pulsotypes. The isolates from non-admitted patient was in the same pulsotype as these food poisoning isolates from Japan. There was 4 isolates from stool which were in the same pulsotypes.

Pulsotyes of *C. perfringens* of positive and negative PCR strains.

Pulsotypes of enterotoxin gene positive and negative *C. perfringens* were shown in table 13. Among the 6 *cpe*-positive strains, they were differentiated into 4 different pulsotypes while the 99 *cpe*-negative strains were in 84 different types.

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Results

Table 5. *C. perfringens* isolated from stools of diarrhea patients.

Patients	**Total specimens**	**Number of isolation**
Admitted-patients	80	20
Non-admitted patient	1	1

* patient had food p oisoning but required no hospitalization

Table 6. Sporulation in DS medium of *C. perfringens* from different sources.

Isolates	**Total number of tested isolates**	**Number of spore positive isolates in DS medium**	
		At 37 °C	**At 43 °C**
Food and water	80	67	67
Stool of admitted	20	9	9
Stool of Non-admitted patients with acute diarrhea	1	1	1
Stool of patient with food poisoning from Japan			
- isolate no.1 (WA)	1	0	0
- isolate no.2 (WB)	1	1	1
- isolate no.3 (WD)	1	1	1

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Table 6 (Continue)

- isolate no.4 (W35)	1	0	0
- isolate no.5 (W40)	1	1	1
Positive control strain (NCTC 8239)	1	1	1
Negative control strain (ATCC 14367)	1	1	1

Table 7. Heat resistant spore of *C. perfringens* from the different sources.

Isolates	**No. of tested isolates with positive spore**	**No. of isolates with heat resistant spore**
Food and water	67	0
Stool admitted-patients	9	0
Stool of non-admitted patient	1	1
Stool of patient with food poisoning isolates from Japan		
- isolate no.2 (WB)	1	1
- isolate no.3 (WD)	1	1
- isolate no.5 (W40)	1	1
Positive control strain (NCTC 8239)	1	1
Negative control strain (ATCC 14367)	1	0

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Table 8. Enterotoxin detection by reverse passive latex agglutination(RPLA).

Sources of isolates	No. of isolates with positive spore	No. of isolates with positive toxin
Food and water	67	0
Stool of admitted-patients	9	0
Stool of non-admitted patient	1	1
Stool of patient with food poisoning isolates from Japan		
- isolate no.2 (WB)	1	1
- isolate no.3 (WD)	1	1
- isolate no.5 (W40)	1	1
Positive control strain (NCTC 8239)	1	1
Negative control strain (ATCC 14367)	1	0

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Table 9. Detection of α–toxin gene by PCR(α_1, α_2)

Sources of isolates	Total isolates	No. of isolates with positive α-toxin gene
Food and water	80	80
Stool of admitted in-patients	20	20
Stool of non-admitted patient	1	1
Stool of patient with food poisoning isolated from Japan	5	5
Positive control strain (NCTC 8239)	1	1
Negative control strain (ATCC 14367)	1	1

Table 10. Detection of enterotoxin gene by PCR

Sources of isolates	Total isolates	No. of isolates with positive enterotoxin gene	
		by Primer (ent1,2)	by Primer (ent 3,4)
Food and water	80	0	0
Stool of admitted-patient	20	0	0
Stool of non-admitted patient	1	1	1
Stool of patient with food poisoning isolate from Japan	5	5	5
Positive control strain (NCTC 8239)	1	1	1
Negative control strain (ATCC 14367)	1	0	0

Table 11. Pulsotypes of *C. perfringens* isolated from different sources.

Sources / Pulsotypes	Food(41)	Water(39)	Stool 1(20)	Stool 2 (1)	Food poisoning(5)
1	1	-	-	-	-
2	1	-	-	-	-
2A	1	-	-	-	-
3	1	-	-	-	-
4	1	-	-	-	-
5	1	-	-	-	-
6	1	-	-	-	-
7	1	-	-	-	-
7A	-	-	1	-	-
8	1	-	-	-	-
8A	-	1	-	-	-
9	1	-	-	-	-
10	1	1	-	-	-
11	1	-	-	-	-
11A	1	-	-	-	-
12	1	-	-	-	-
13	1	-	-	-	-
14	1	-	-	-	-
15	2	-	-	-	-
16	1	-	-	-	-

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Table 11 (Continue)

Pulsotypes	Food(41)	Water(39)	Stool 1(20)	Stool 2 (1)	Food poisoning(5)
17	1	-	-	-	-
18	2	-	-	-	-
19	1	-	-	-	-
20	1	-	-	-	-
21	2	-	-	-	-
22	1	-	-	-	-
23	1	-	-	-	-
24	1	-	-	-	-
25	1	-	-	-	-
26	1	-	-	-	-
27	1	-	-	-	-
27A	-	1	-	-	-
28	1	1	-	-	-
29	1	-	-	-	-
30	1	-	-	-	-
31	1	-	-	-	-
32	1	-	-	-	-
33	1	-	-	-	-
34	1	-	-	-	-

Table 11 (Continue)

Pulsotypes	Food(41)	Water(39)	Stool 1(20)	Stool 2 (1)	Food poisoning(5)
35	1	-	-	-	-
35A	-	-	1	-	-
36	1	-	-	-	-
37	-	1	-	-	-
38	-	1	-	-	-
39	-	1	-	-	-
40	-	1	-	-	-
41	-	1	-	-	-
42	-	1	-	-	-
43	-	1	-	-	-
44	-	1	-	-	-
45	-	1	-	-	-
46	-	2	-	-	-
47	-	1	-	-	-
48	-	1	-	-	-
49	-	1	-	-	-
50	-	1	-	-	-
51	-	1	-	-	-
52	-	1	-	-	-
53	-	1	-	-	-

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Table 11 (Continue)

Pulsotypes	Food(41)	Water(39)	Stool 1(20)	Stool 2 (1)	Food poisoning(5)
54	-	1	1	-	-
55	-	1	-	-	-
56	-	1	-	-	-
57	-	1	-	-	-
58	-	1	-	-	-
59	-	1	-	-	-
60	-	2	-	-	-
61	-	1	-	-	-
62	-	1	-	-	-
63	-	1	-	-	-
64	-	1	-	-	-
65	-	1	-	-	-
66	-	1	-	-	-
67	-	1	-	-	-
68	-	1	-	-	-
69	-	1	-	-	-
70	-	-	1	-	-
71	-	-	1	-	-
72	-	-	1	-	-
73	-	-	1	-	-

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Table 11 (Continue)

Pulsotypes	Food(41)	Water(39)	Stool 1(20)	Stool 2 (1)	Food poisoning(5)
74	-	-	1	-	-
75	-	-	1	-	-
76	-	-	1	-	-
77	-	-	2	-	-
78	-	-	1	-	-
79	-	-	1	-	-
80	-	-	1	-	-
81	-	-	1	-	-
82	-	-	1	-	-
82A	-	-	1	-	-
83	-	-	1	-	-
84	-	-	1	-	-
85	-	-	-	1	2
86	-	-	-	-	1
87	-	-	-	-	1
88	-	-	-	-	1

[1]stool form admitted patient

[2]stool from non-admitted patients

( ) = number in the blanket indicates the number of isolates from each source

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Table 12 Similar pulsotypes from different sources

Pulsotype	Sources
2, 2A	food, food
7, 7A	food, stool
8, 8 A	food, water
10, 10	food, water
11, 11A	food, food
15, 15	food, food
18, 18	food, food
21, 21	food, food
27, 27A	food, water
28, 28	food, water
35, 35A	food, stool
46, 46	water, water
54, 54	water, stool
60, 60	water, water
77, 77	stool, stool
82, 82A	stool, stool
85, 85, 85	stool, food poisoning, food poisoning

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Table 13. Pulsotypes of enterotoxin gene positive strains

Pulsotypes of *C. perfringens*	
***Cpe* gene positive**	***Cpe* gene negative**
85, 86, 87, 88	1, 2, 2A, 3, 4, 5, 6, 7, 7A, 8, 9, 10, 11, 11A, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 27A, 28, 29, 30, 31, 32, 33, 34, 35, 35A, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 82A, 83, 84

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Figure 6. *C. perfringens* α-toxin gene amplification from various sources. Lane 1 Marker, lane 2-4 isolate from food, lane 5-6 isolates from stool, lane 7-8 isolates from water and land 9 positive control (NCTC 8239), lane 10 negative control (ATCC 14367)

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Figure 7. Enterotoxin gene (250 bp) amplification.

Lane 1-Marker lane 2-6 (WA, WB, W35, W40, WD)

lane 7-8 isolates from stool, lane 9 positive control

(NCTC 8239), lane 10 negative control(H_2O)

1 2 3 4 5 6 7 8 9 10

Figure 8. Enterotoxin gene (163 bp) amplification.

Lane 1 Marker, lane 2-6 (WA, WB, W35, W40, WD),

lane 7 isolates from stool, lane 8-9 positive control (NCTC 8239)

lane 10 negative control(ATCC 14367)

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1 2 3 4 5 6 7

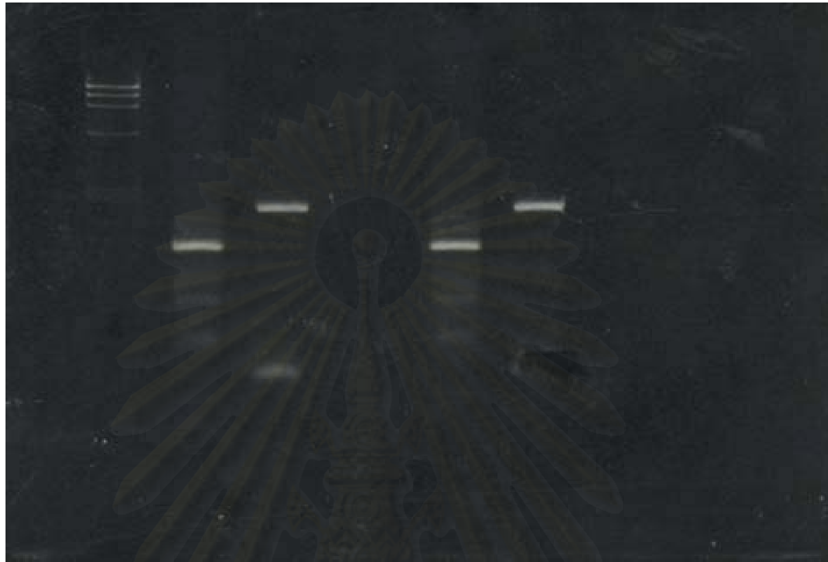

Figure 9. Enterotoxin gene (163 bp and 250 bp) amplification. Lane 1 Marker, lane 2-3 isolate from stool from non-admitted patient, lane 4 negative control. (ATCC 14367), lane 5-6 positive control (NCTC 8239), lane 7 negative control (H_2O)

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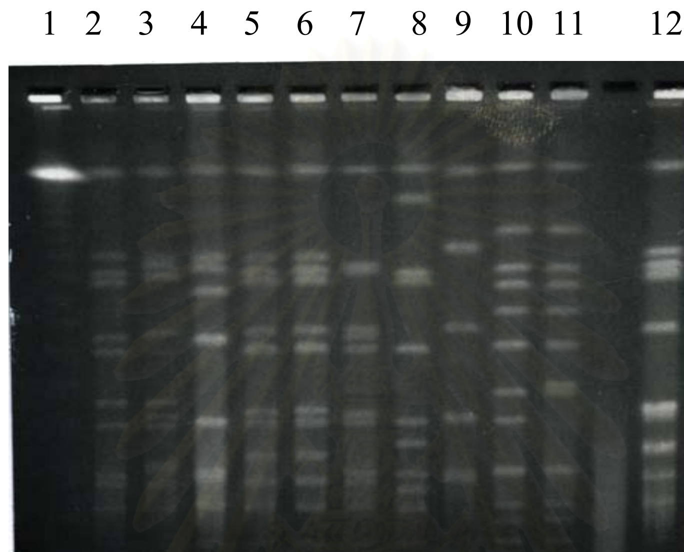

Figure 10. Pulsed – field gel electrophoresis with a contour-clamped homogenous electric field (CHEF) of DNA of *C. perfringens*. DNA were digested with *Sma* I, and electrophoresis condition consisted for 24 h at 200 V. Lane 1 : standard lambda ladder marker; Lane 2, 3, 10, 11, 12 : DNA of *C. perfringens* isolated from stools; lane 4-9 : DNA of *C. perfringens* isolated from food and water.

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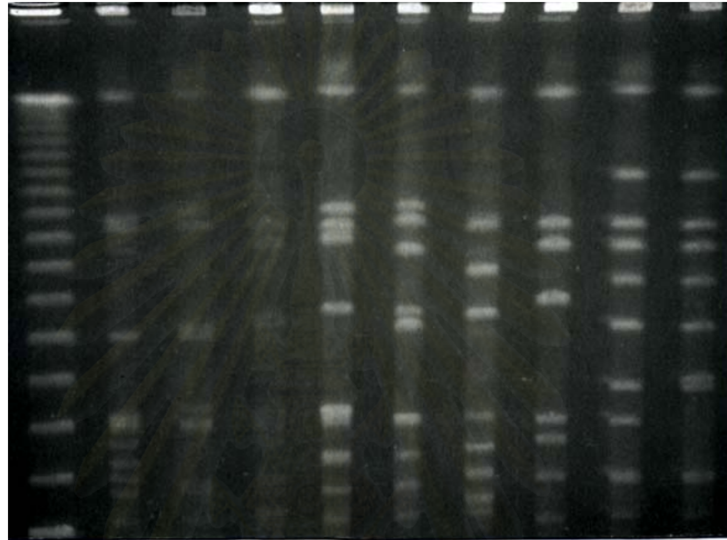

Figure 11. Pulsed-field gel electrophoresis with a contour-clamped homogenous electric field (CHEF) of DNA of *C. perfringens*. DNA were digested with *Sma* I, and electrophoresis 200 V. for 24 h. Lane 1 : standard lambda ladder marker ; lane 2-10 : DNA of *C. perfringens* isolated from stools

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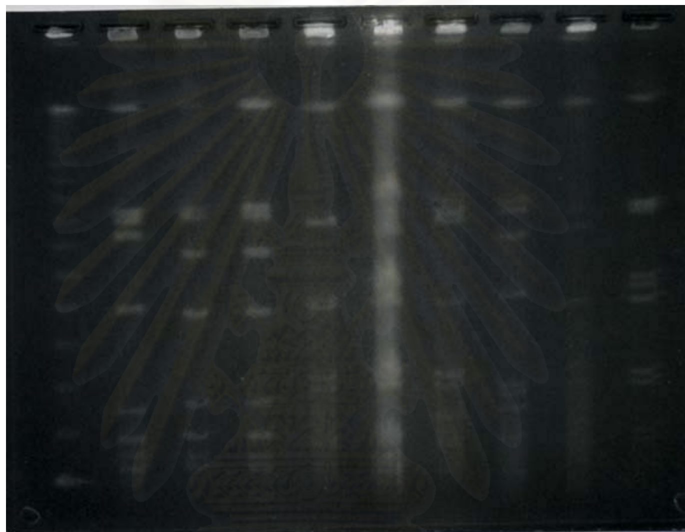

Figure 12. Pulsed-field gel electrophoresis with a contour-clamped homogenous electric field (CHEF) of DNA of *C. perfringens*. DNA were digested with *Sma* I, and electrophoresis.

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Figure 13 Diagramatic pattern of 88 pulsotypes of *C.perfringens*

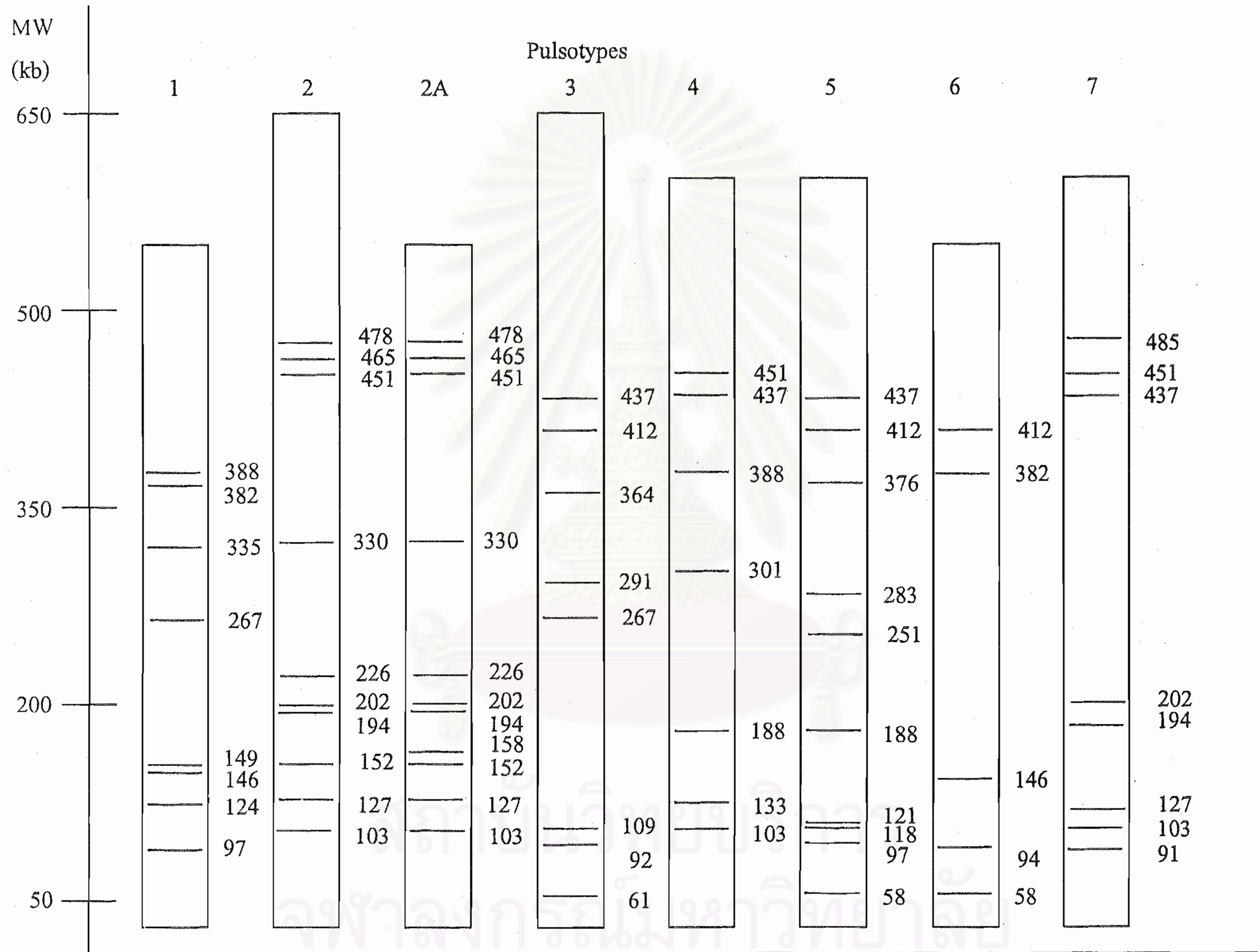
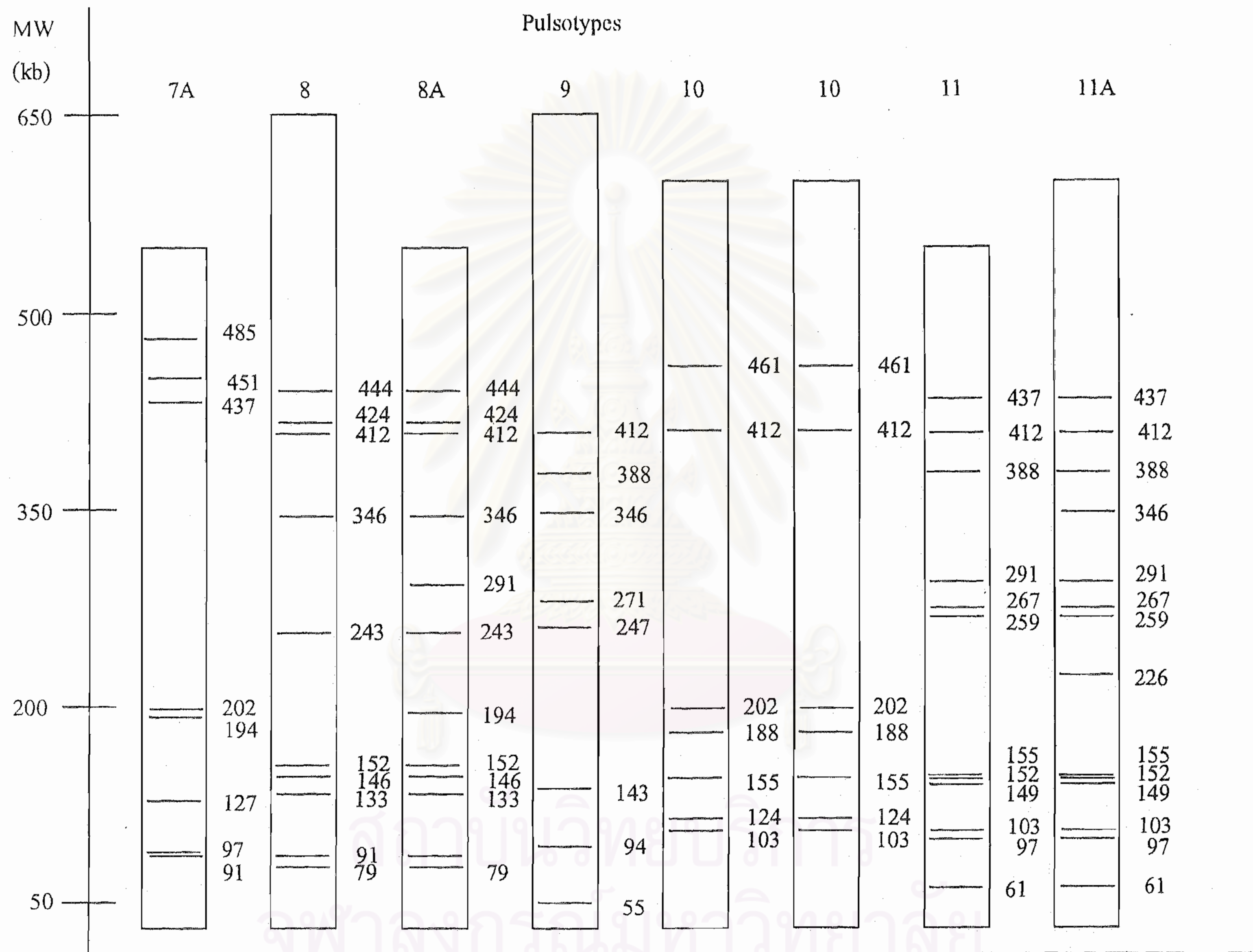


Figure 13 (continue)



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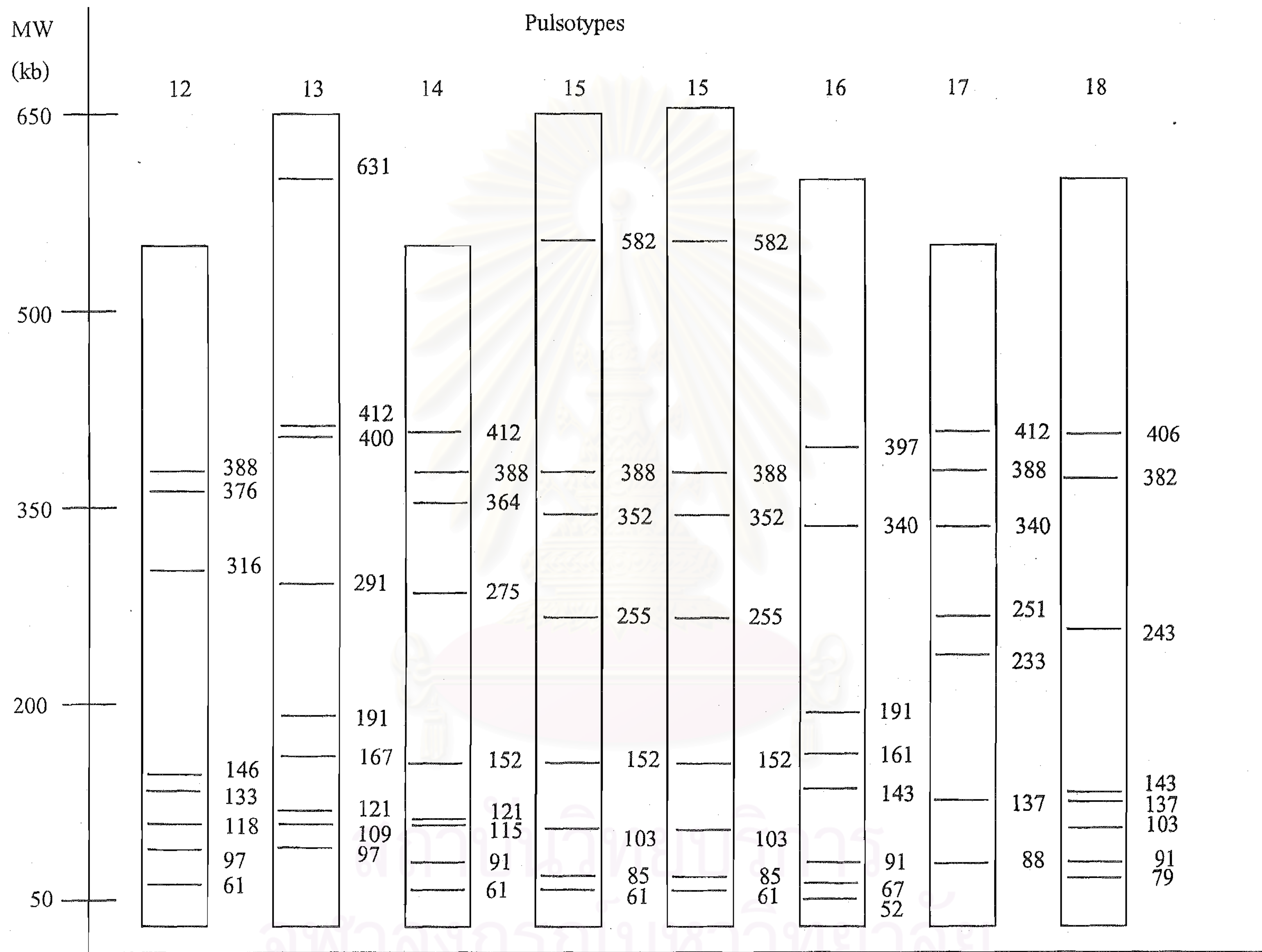

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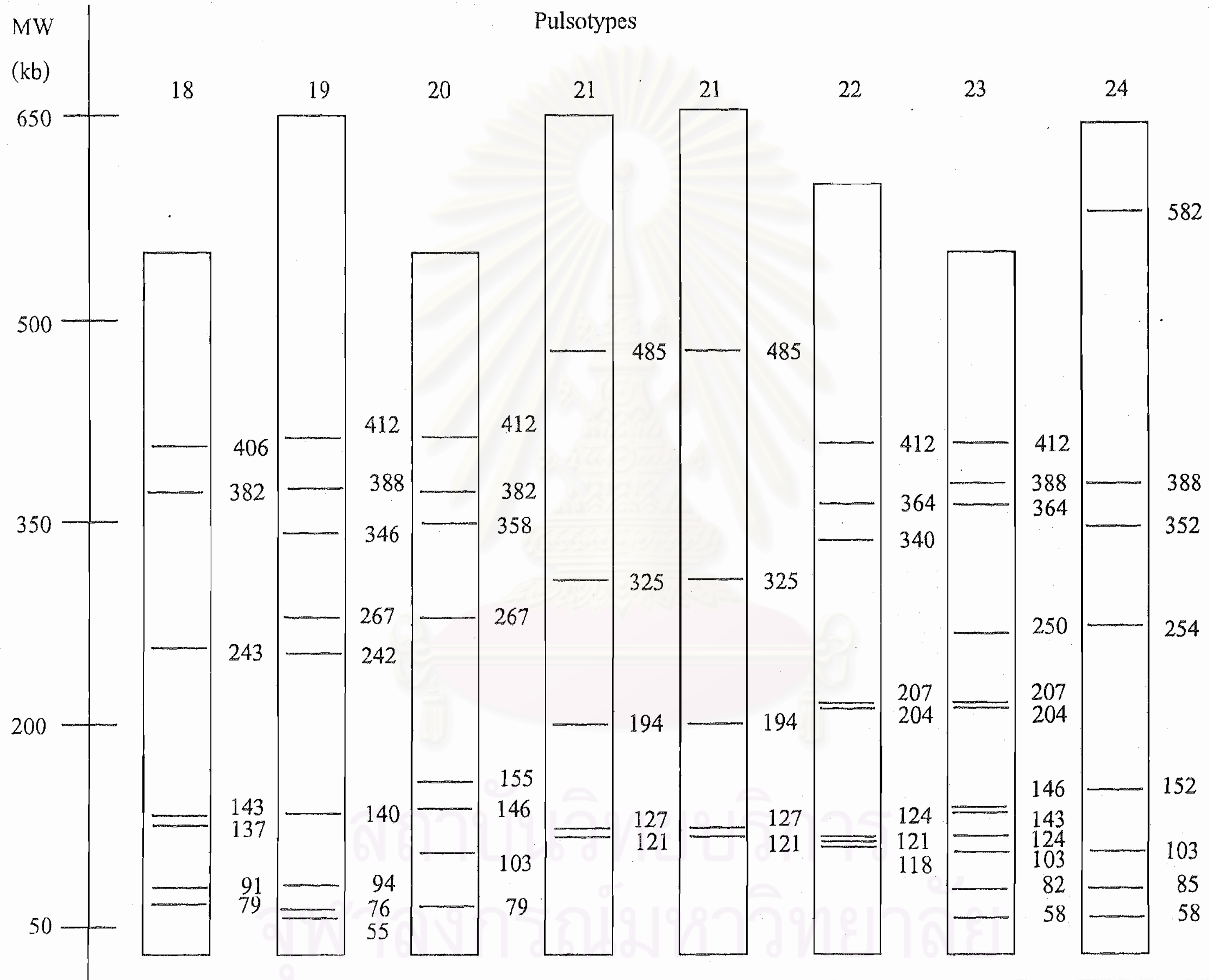

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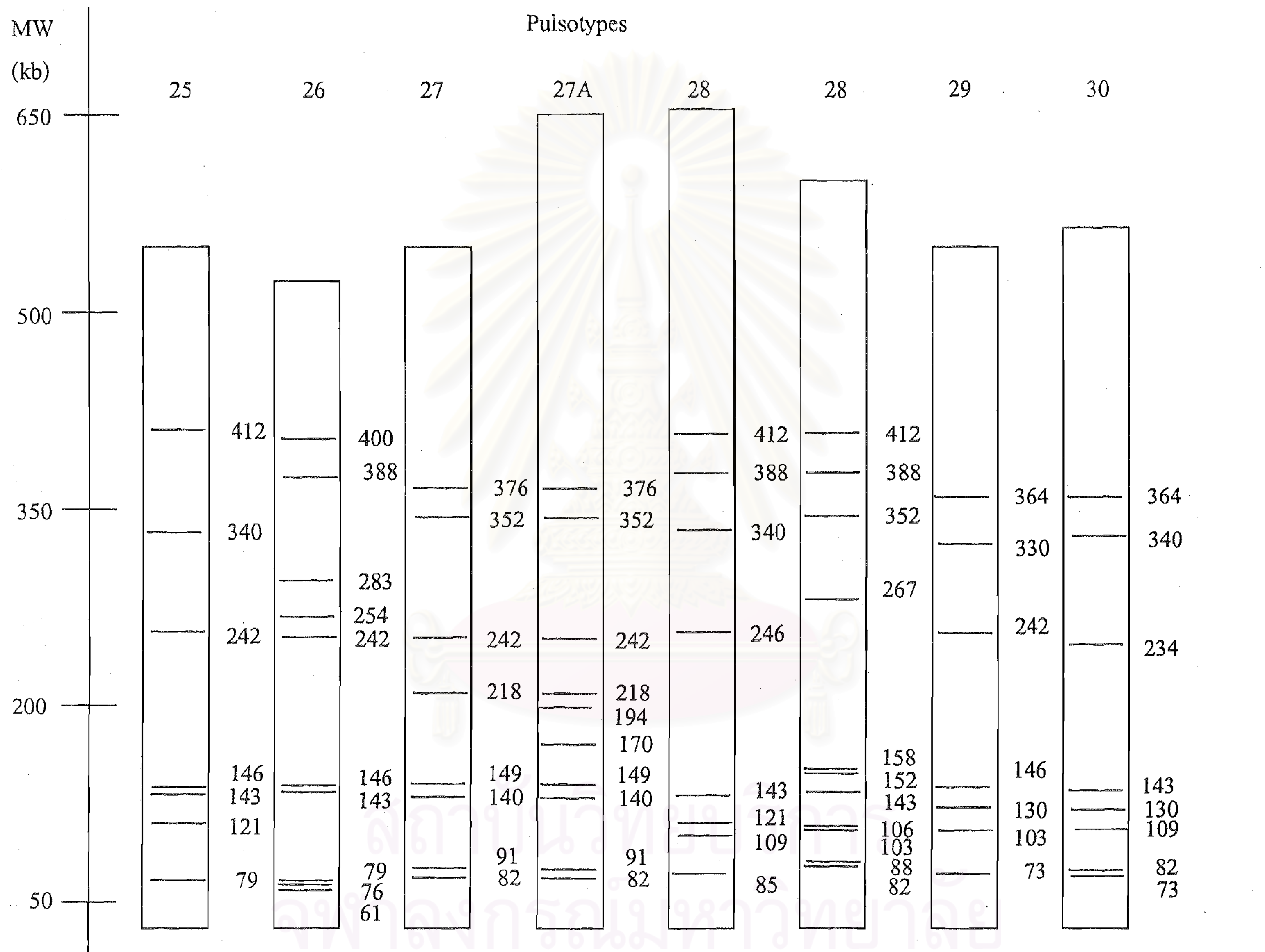

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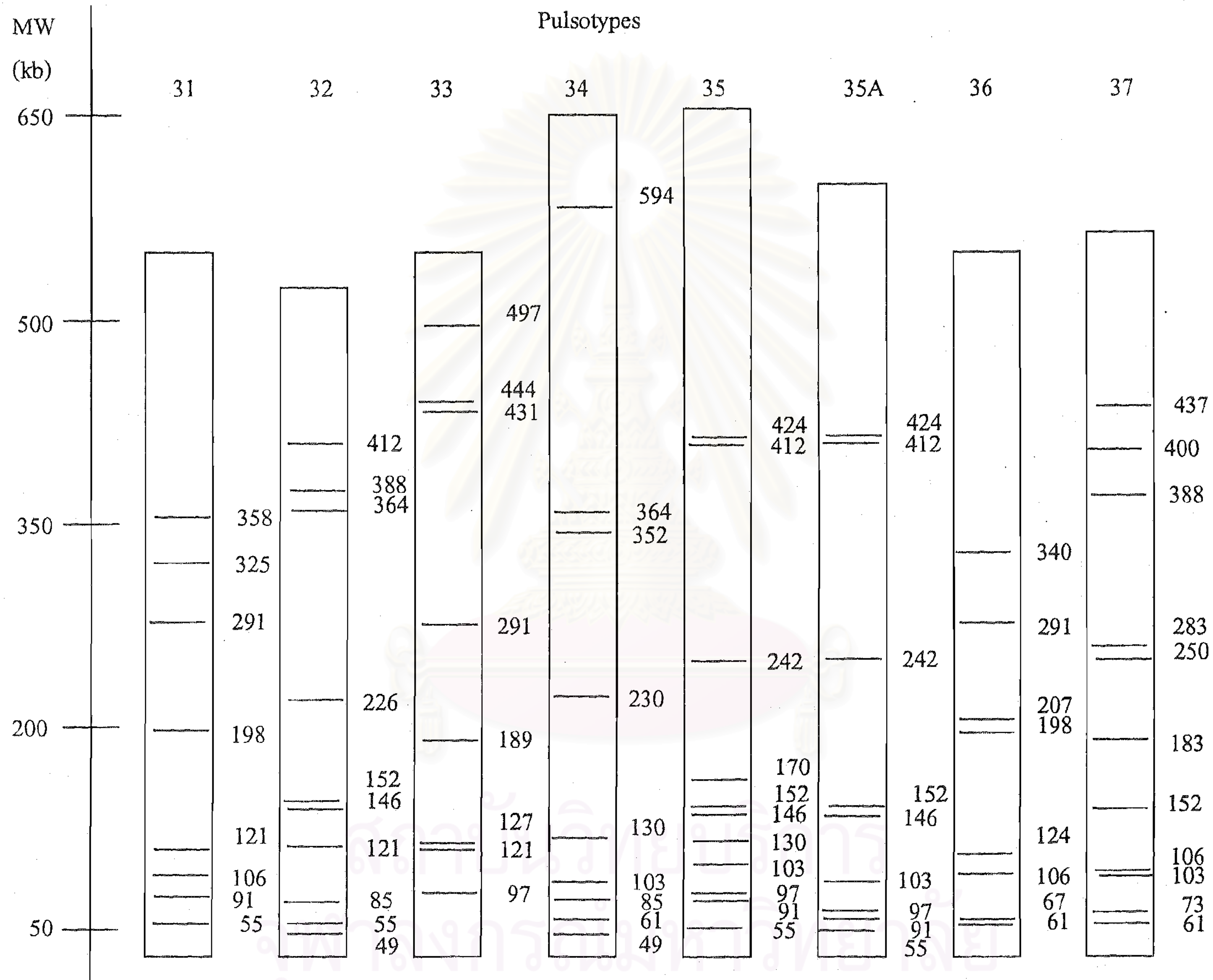

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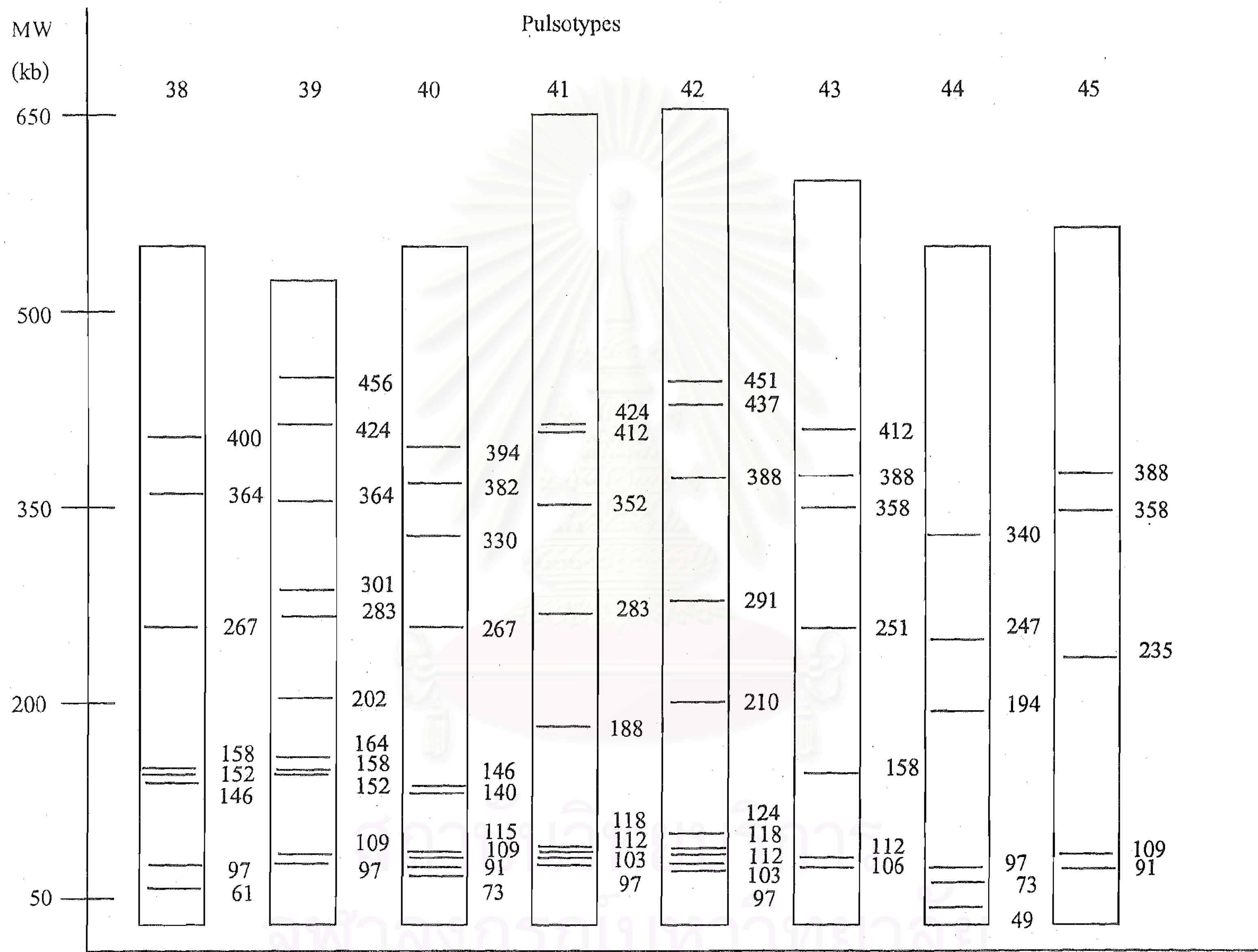

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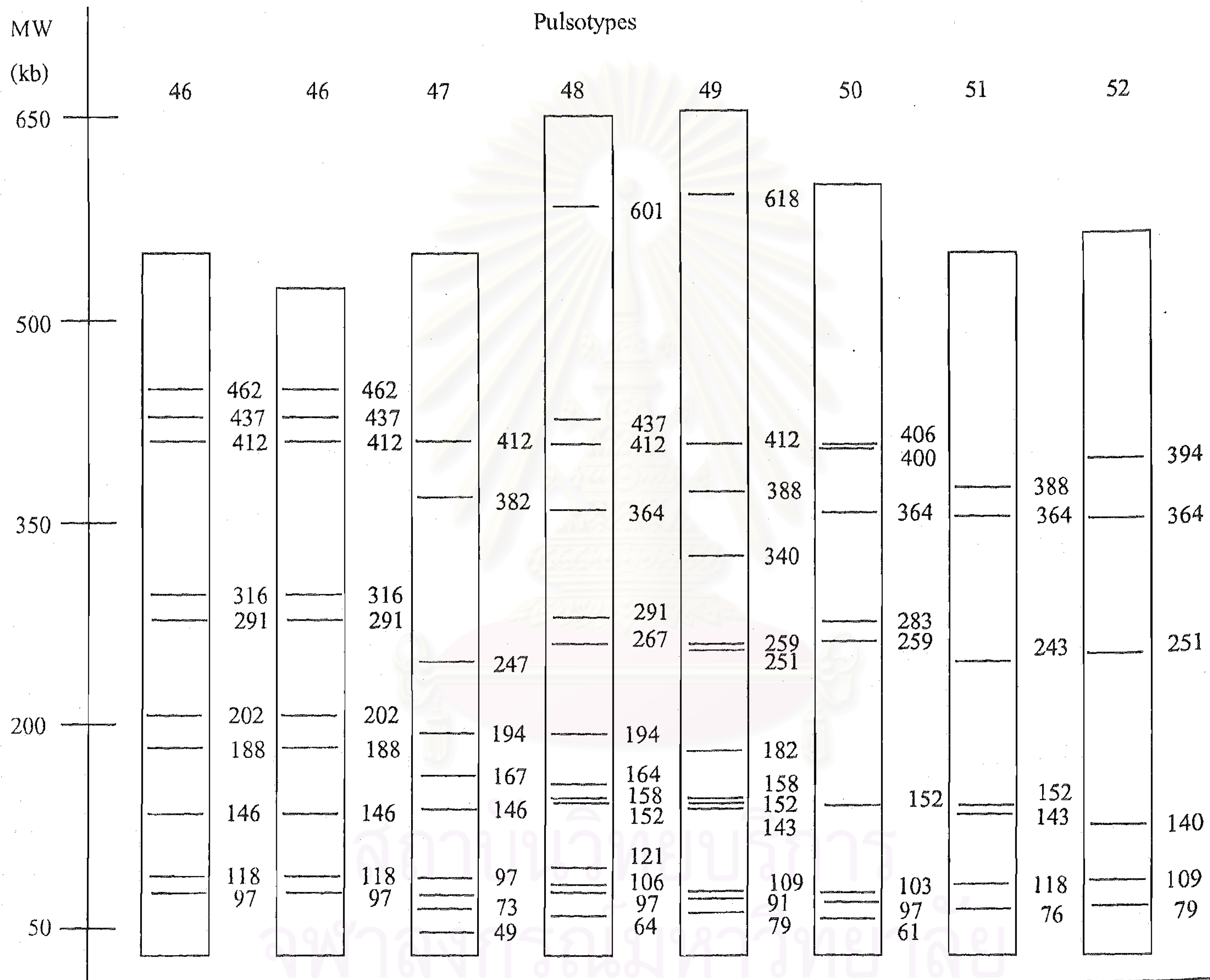

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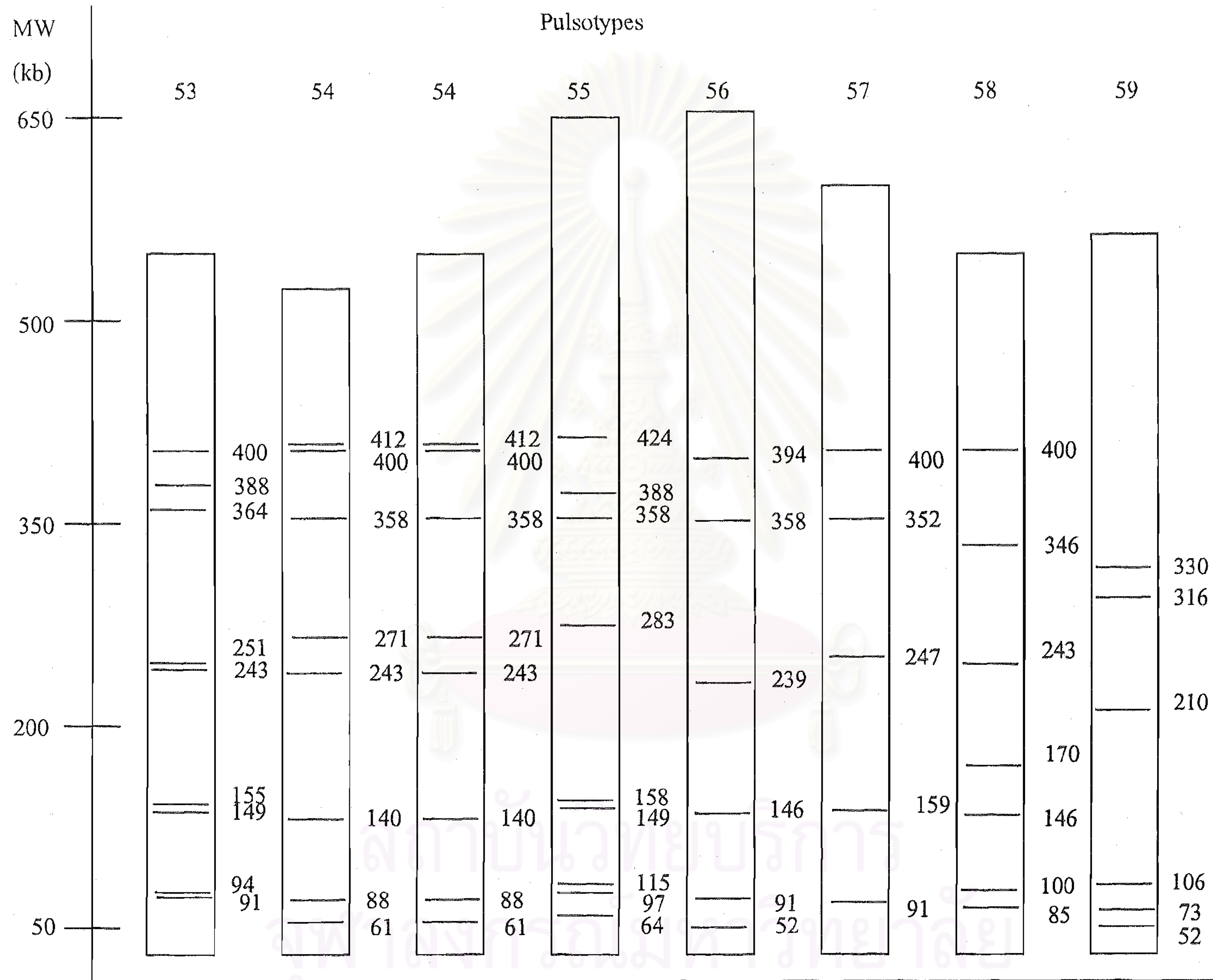

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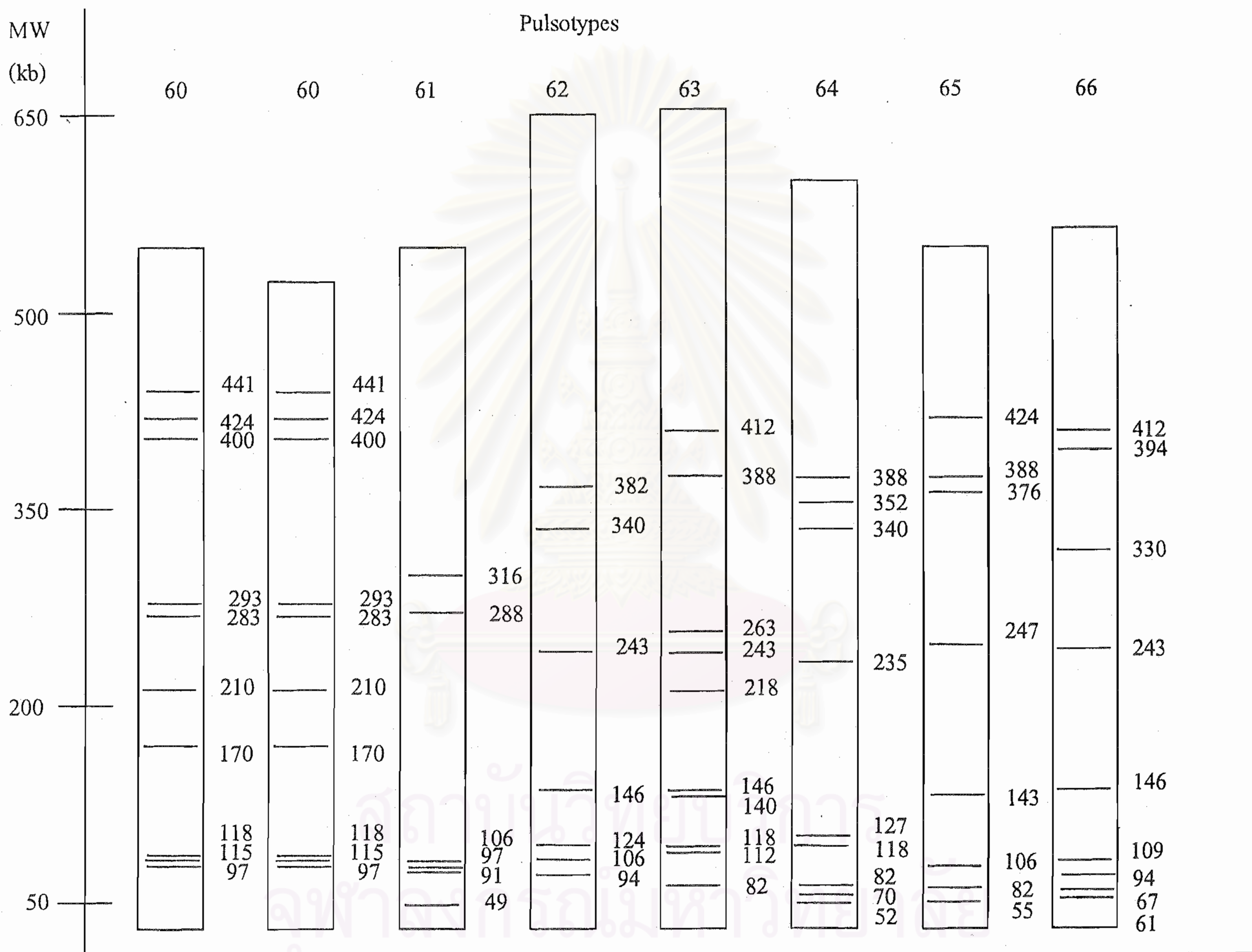

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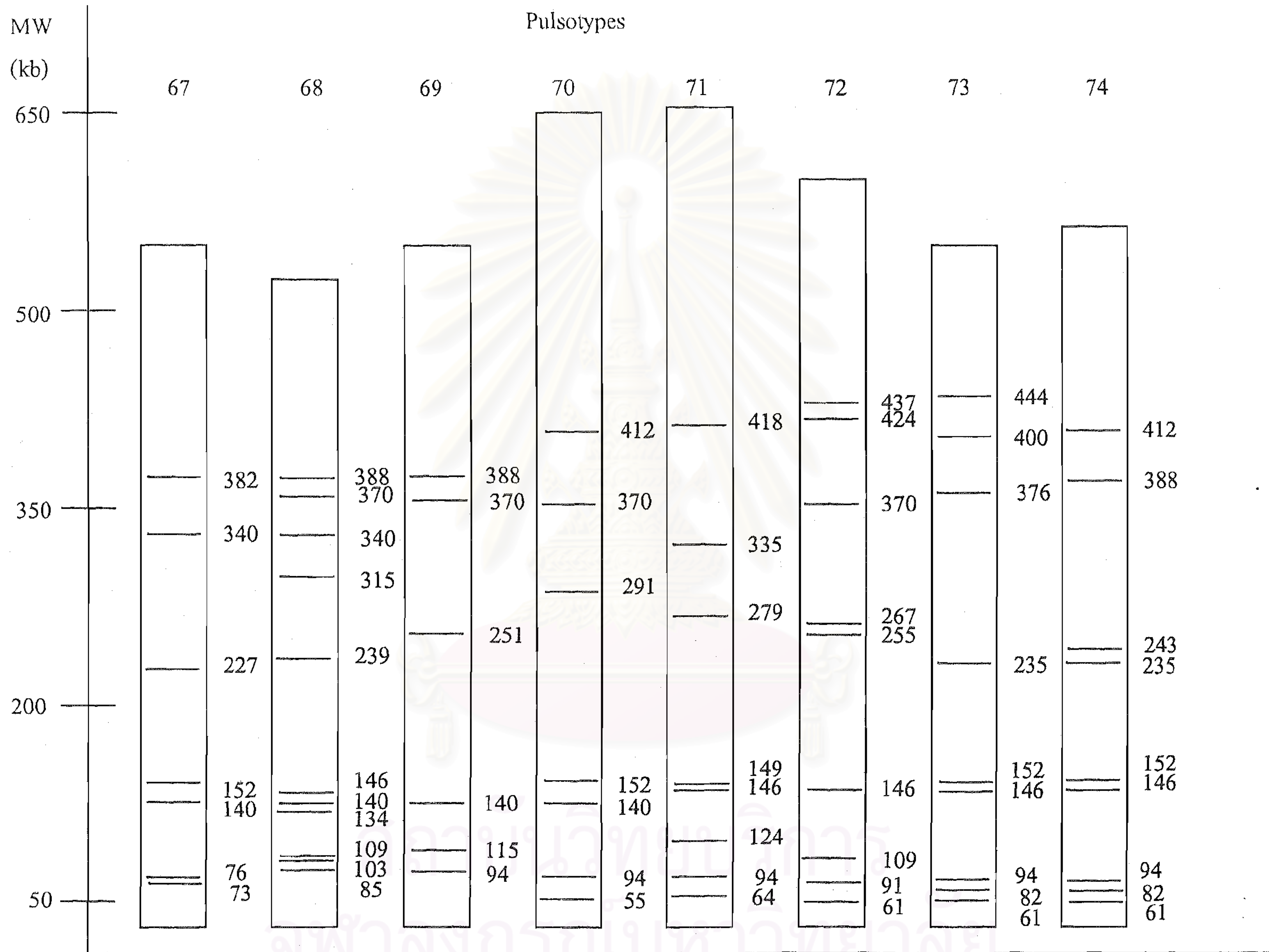

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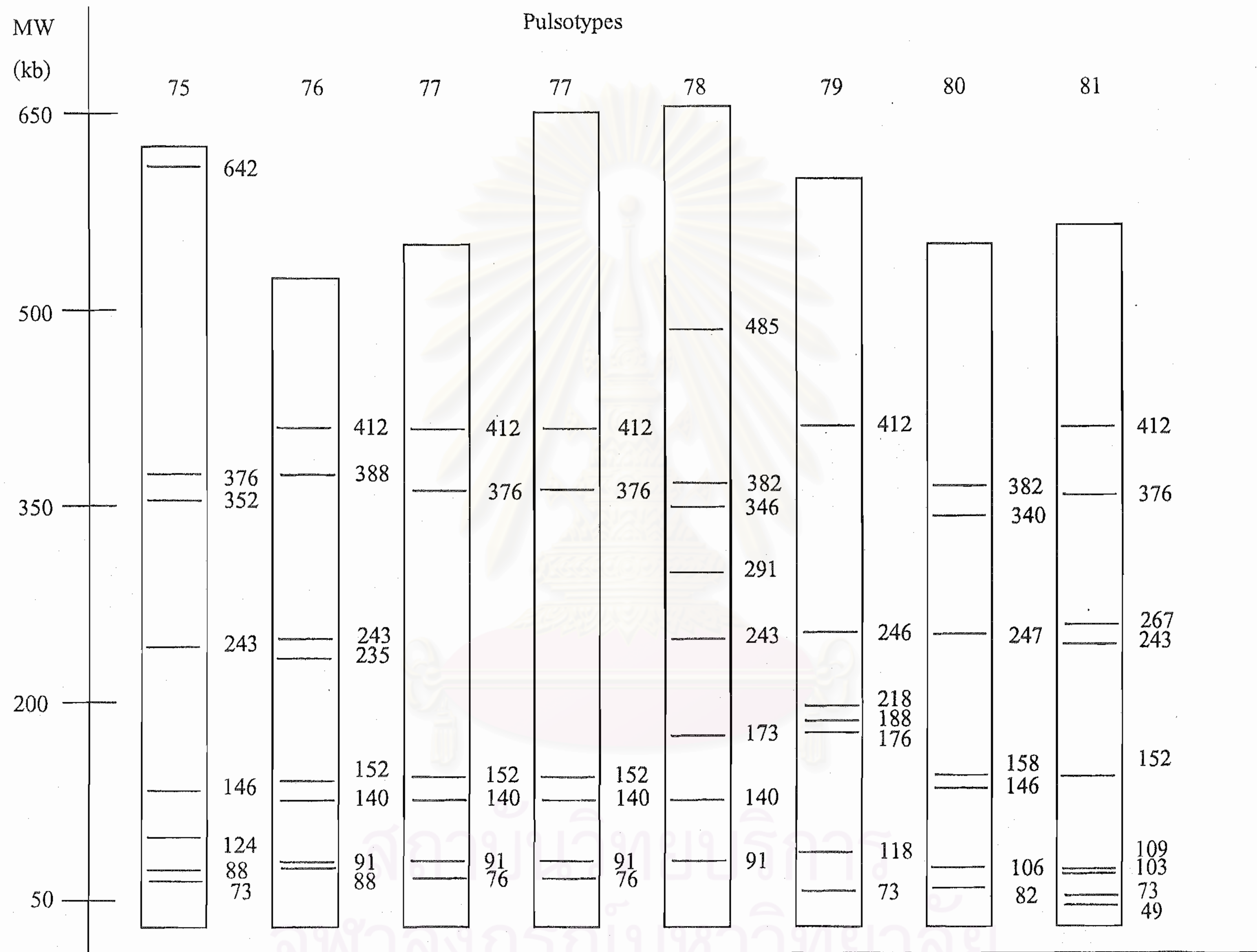

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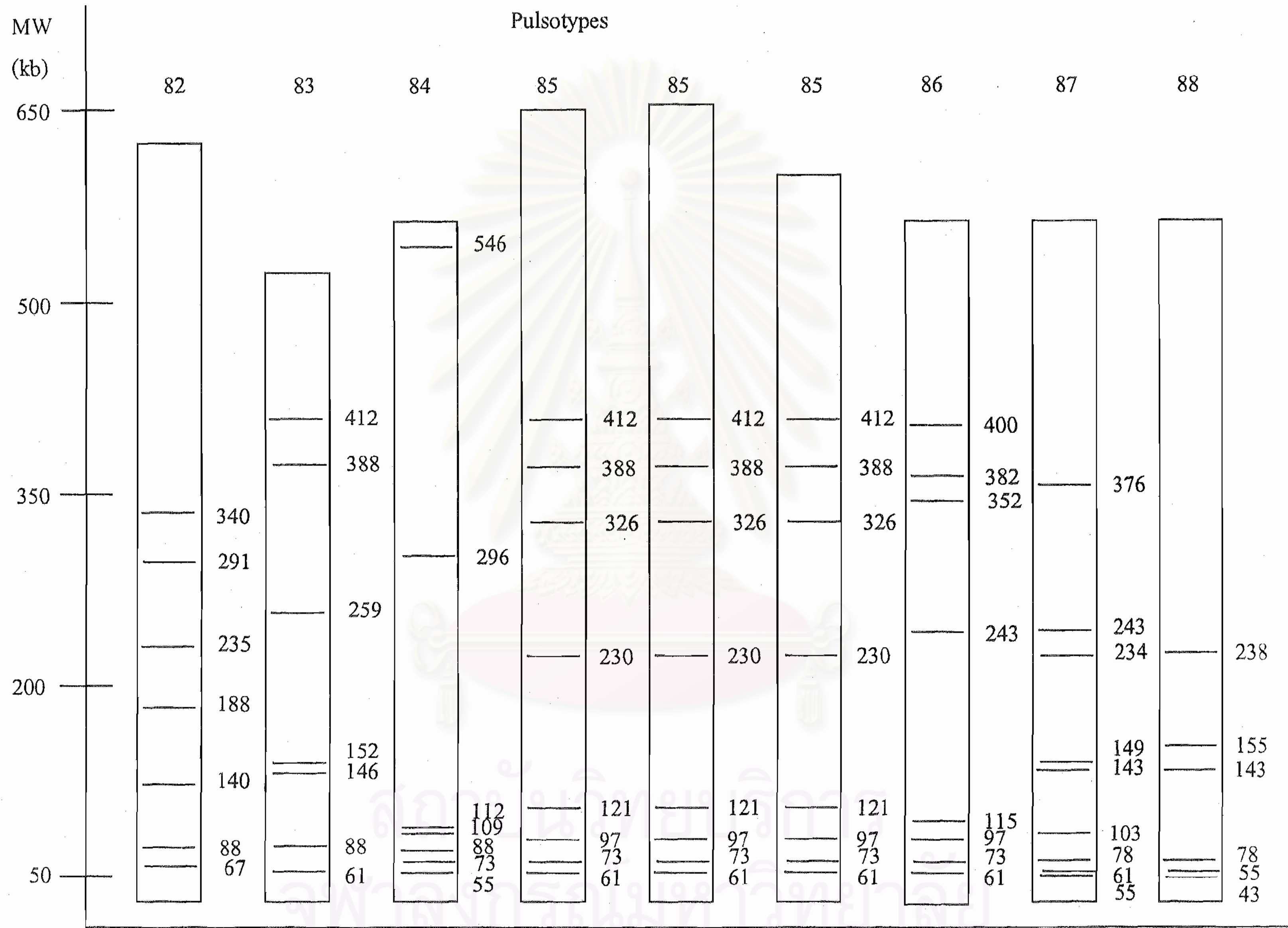


CHAPTER VI

Discussion

In general, *C. perfringens* enterotoxin (CPE) is produced only during the sporulation phase. From the total of 106 *C. perfringens* isolates only 80 strains could sporulate (76%). The Duncan-Strong (DS) medium which has been wildly recommended sporulating medium for *C. perfringens* was used in this study. Harman et al (1986)(101) used DS medium to sporulate 24 strains of *C. perfringens* isolated from stools of food poisoning patients and was compared this medium with other media. They showed that DS medium was the best medium in which all isolates could sporulate. Even though, most of the isolates from food and water in this study could sporulate but about half of the isolates from stool of the admitted-patients and two out of five isolates from food poisoning in Japan could not sporulate in Ds medium.

Rapidly metabolizable carbohydrates such as glucose are generally avoided in sporulation media because they are vigorously fermented by certain saccharolytic species of clostridium, resulting in considerable acid production which overproduction of acid end-products which killed the vegetative cells before sporulation could occur but in DS medium soluble starch is used as an energy source. Sporulation of *C. perfringens* could occur when the medium pH was adjusted to 7.8 with sodium carbonate(102-104).

For the optimum temperature, most investigator suggested that 37 $^{\circ}$C was appropriate for *C. perfringens* sporulation. However there still had some

limitation for several isolates. Carcia-Alvarado et. al. (1992) suggested that at 43 °C most of the test strains in their study could sporulate more than at 37 °C. However the result this not agree with their study because no different in the sporulation was observed.

Although sporulation is generally initiated by a lack of nutrients, but clostridia need an energy source for macromolecular synthesis during sporulation and the cells may die if one or more nutrients are completely absent. However, not all Clostridium species can sporulate well in laboratory media because conditions for their sporulation can be highly specific. It has been mentioned that enterotoxin producing strains must be able to produce heat resistant spore(105)

In this study heat resistant spore produced by *C. perfringens* from different sources were investigated. It has been shown by Skjelkvale (58) that *C. perfringens* with heat resistant spore was related to food poisoning because of its ability to survived from heat used in the cooking process. It was clearly shown that all 3 food poisoning isolated from Japan could produced heat resistant spore which was agreeable with the previous study by Skjelkvale and Takashi (1977). In addition one isolated from non-admitted patient with acute diarrhea also produced heat resistant spore indicating that this patient could possibly be infected with food poisoning strain while the other sporulating isolates from in-patients did not produce heat resistant spore. Unfortunately, during the time of specimen collection in this study, more stool specimens from non-admitted patients stool specimen from non-admitted patients with acute diarrhea could not be obtained accept only one specimen.

In fact, diarrhea symptoms of *C. perfringens* food poisoning self-limited so the patients with this diarrheal disease are normally not admitted patients. However, at present there have been several studies indicated *C. perfringens* could possibly be one of the causative agents for nosocomial diarrhea (107-108). Therefore, this study of *C.perfringes* nosocomial diarrhea in admitted-patient were also one of the objective of study.

There have been several methods for enterotoxin producing *C. perfringens* detection as already described in Chapter IV. Fach (1997) used latex agglutination test to detect enterotoxigenic *C. perfringens* in food and fecal samples. They as well as many other investigators found problem that *C. perfringens* did not sporulate in sporulating media used in those studies (56, 106, 111). The limitation of reverse passive latex agglutination is that the detection of enterotoxin depended only on the sporulation ability of the organism. However, in this study latex agglutination test kit (RPLA) was also used comparison. It was shown that enterotoxin could be detected from only one sporulating isolate from stool of non-admitted patient and three sporulating food poisoning isolates from Japan by RPLA kit as well as positive control strains (NCTC 8239). This result confirmed the results from previous study (62, 67, 69) that RPLA kit detected enterotoxin only from sporulating *C. perfringens.*

Alpha-toxin (α-toxin) is commonly produced by all *C. perfringens* types (1). The success of DNA isolation in this study was supported by the amplification of α-toxin gene from all *C. perfringens* isolates. The enterotoxigenic *cpe* gene were obtained by the use of two pair of primers

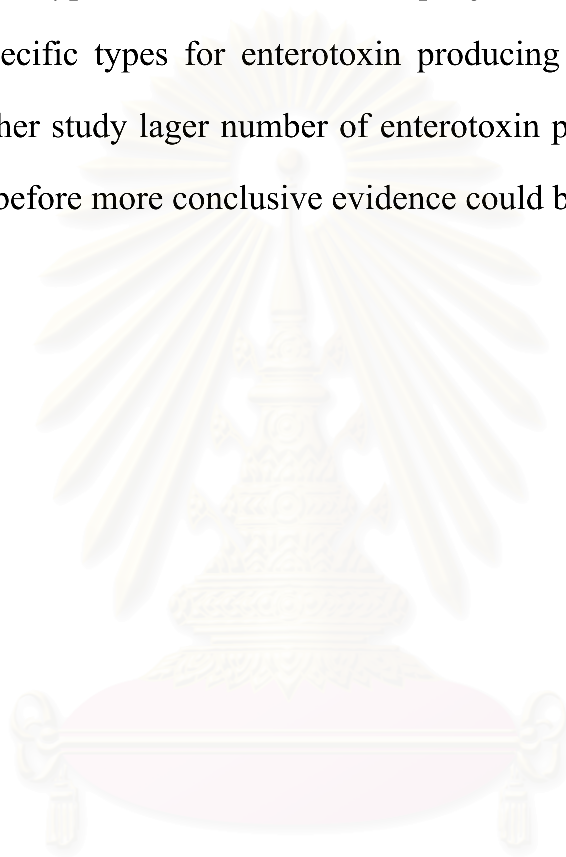
(ent$_1$, ent$_2$ and ent$_3$, ent$_4$). No *cpe* gene was demonstrated in all isolates from food, water and stool of admitted patients which was similar to the result showed by the used of RPLA technique. However, the *cpe* gene were detected from 5 *C. perfringens* from the Japan strains, 1 of the non-admitted patient and positive control strain but enterotoxin could not be detected from the two non spore producing isolates by using RPLA and the detection of heat resistant spore.

This part of the study confirmed that the detection of *cpe* gene by PCR is the more effective technique for detection of enterotoxigenic isolates of *C. perfringens* than other technique including RPLA as already mention in the study by Kokai-Kun and Fach (56, 106)

The typing of pulsed-field gel electrophoresis (PFGE) has been successfully use to discriminate all isolates into 88 different pulsotype. This technique was easy to perform and provided good discriminatory power and reproducibility all *C. perfringens* isolated from different sources was also inclced in this study. Similar pulsotypes was observed in same isolates from different sources such as food-water, food-stool, food-other food, stool-other stool. However, almost all isolates from food and water were in the many distinct clones indicated that there was o major type of *C. perfringens* among water and food samples collected by Food Division of Public Health during 1995-1999. It was suggested chat no food-poisoning outbreak had occur during the time of specimen collection. Interestingly, the toxigenic isolate from only one Thai non-admitted patient was in the same pulsotype as tow of the food-poisoning outbreak isolates from Japan (pulsotype 85). It could

suggested that *C. perfringens* type 85 from this subtype could be one of major food poisoning isolates.

In addition, the result also showed that *C. perfringens* with *cpe* gene were in different types from those without *cpe* gene. There could possibly be the limited specific types for enterotoxin producing *C. perfringens* strains. However, further study lager number of enterotoxin producing strains should be performed before more conclusive evidence could be made.



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CHAPTER VII

Conclusion

The prevalence of the enterotoxin gene in 106 *C. perfringens* samples isolates from 80 food and water, 20 stool samples from admitted-patients with acute diarrhea, 1 sample from non-admitted patient with acute diarrhea and 5 food poisoning isolates from Japan were determined by PCR. Enterotoxin from all isolates were detected by using reverse passive latex agglutination (RPLA). All 4 spore positive isolates produced enterotoxin. (None of the isolates from food and water and from stool of the admited-patients with acute diarrhea had enterotoxin gene. The isolate from stool of one non-admitted patient with acute diarrhea as well as all the 5 food poisoning isolates from Japan had *cpe* gene.)

Pulse-field gel electrophoresis (PFGE) was used to perform genotyping of all *C. perfringens* isolates. This method was easy to performe and reproducible. All isolates were discriminated into 88 different pulsotypes. The PFGE patterns demonstrated considerable diversity among all isolates according to the Ridell et. al. (1998) interpretation procedures by PFGE. The pulsotype of the *cpe* gene positive isolate from non-admitted Thai patient was the same as a *cpe* gene positive isolates from Japan.

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APPENDIX I

Media, Chemical agents, Materials and Instruments

Media

1. Brain Heart infusion broth (BHI)

Calf brains, infusion form	200	g
Beef heart, infusion form	250	g
Proteose peptone	10	g
Bacto dextrose	2	g
Sodium Chloride	5	g
Disodium phosphate	2.5	g

To redehydrate, dissolve 37 grams in 1 liter distilled or deionized water. Dispense as desired and sterilize in the autoclave for 15 minutes at 15 pound pressure. Final pH 7.4 at 25°C

2. Brain heart infusion agar (BHIA)

The same as 1 but add Bacto agar 15 g. Suspend 52 grams in 1 liter distilled or deionized water and boil to dissolve. Sterilize at 121-124°C for 15 minutes. Final pH 7.4 at 25°C

3. Sheep blood agar

Prepare BHIA, after sterilization allow it cools to 50°C and add 5% sheep blood in the media.

4. Egg yolk agar

Proteose peptone	40.0	g
Na_2Hpo_4	5.0	g
KH_2PO_4	1.0	g
NaCl	2.0	g
$MgSO_4$	0.1	g
Glucose	2.0	g
Hemin solution (5 mg/ml)	1.0	ml
Agar	20.0	g
Distilled water	1000.0	ml

Mix well, adjust pH to 7.6 Boil to dissolve. Dispense 20 ml/tube. Autoclave at 118°C for 15 minutes. Cool to 50°C. Add 2 ml inolex egg yolk emulsion. Mix and pour plates.

5. Cooked Meat Medium

Meat	500	g
Nutrient broth base (pH 7.4)	10	ml
Distilled water	1000	ml

To 1 litre boiling distilled water add 500 g minced fresh meat and simmer for 1 h. Drain off the liquid and partly dry the cooked minced meat,

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first by pressing between layers of cloth. Place the mince in 150 mm. Test tubes. Cover the meat with 10-12 ml nutrient broth base (pH 7.4) and autoclave at 121°C for 10 min.

6. Indole nitrate medium

Reagent A

Sluphanillic acid	0.5	g
Glacial acetic acid	30.0	ml
Distilled water	120.0	ml

Reagent B

1-napthylamine-7 sulphonic acid (cleve's acid)	0.2	g
Glacial acetic acid	30.0	ml
Distilled water	120.0	ml

Add the water to the cleve's acid and warm in a water bath. Filter, cool and add the acetic acid.

Add 2 ml reagent A followed by 2 ml reagent B to a 5 ml volume of the culture. Mix, allow to stand for 10 min and read.

7. Glycerol broth (stock anaerobic)

Tryptose	10.0	g
NaCl	5.0	g
Beef extract	3.0	g
Yeast extract	5.0	g
Cysteine HCL	0.4	g

Glucose	1.0	g
Na_2HPO_4	4.0	g
Glycerol	150.0	ml
Distilled water	850.0	ml

Combine all the ingradients . Add 2 ml per tube and sterilize in the autoclave for 15 minutes at 15 pounds pressure.

8. DS medium

Yeast extract (Oxoid L21)	4.0	g
Proteose peptone (Oxoid L85)	15.0	g
Soluble starch	4.0	g
Sodium thioglycollate	1.0	g
$Na_2HPO_4.7H_2O$	10.0	g
Distilled water	1000.0	ml

Autoctave at 121°C for 15 minutes. Add a sufficient amount of filter-sterilised 0.66 M sodium carbonated to increase the pH to 7.8±0.1.

9. Malachite green (5% agueous)

Malachite green	5	g
Distilled water	95	ml

Mix well, filter before used.

Chemical agents

Strip (Sigma, USA)

McFarland No. 3, 4 (bio Merieux)

Tris (Bio Rad, USA)

Boric acid (Merck, Germany)

EDTA (Sigma, USA)

NaCl (Merck, Germany)

Sarkosyl (Sigma-USA)

Ammonium persulfate (Merck-Germany)

Low melting agarose (Difco, USA)

Sodium dodecyl sulfate (Difco, USA)

PMSF (Sigma, USA)

Mutanolysin (Amresco, USA)

30% acrylamid /bis-acrylamide (Bio-Rad, USA)

dNTP (Promega, USA)

10x $MgCl_2$ free buffer (Promega, USA)

25 M $MgCl_2$ (Promega, USA)

6x loading dye (Promega, USA)

10x REB (Bio Lab, New England)

Sucrose (Sigma, USA)

HCI (Merck, Germany)

Deoxynucleoside triphosphate (Bio Rad, USA)

Mineral oil (Sigma, USA)

Ethidium bromide (Bio Rad, USA)

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TEMED (Bio Rad, USA)

Restriction enzyme *Sma* I buffer (Bio Rad, USA)

Lambda ladder (Bio Rad, USA)

Meterials

1.5 ml microtube (Pyrex, USA)

10 ml snap-top tube (Pyrex, USA)

5 ml snap-top tube (Pyrex, USA)

15 ml round bottom tube, screw cap (Pyrex, USA)

Plug mold (Bio Rad, USA)

Eppendorf microcentrifuge

Micropipet

Tip

Cotton swab

Cylinder

Test tube

X-ray film cassettes

Pasture piper

Gel block

Gas Pak (Oxoid, England)

Palidium

Instruments

ATB electronic pipette (Bio Rad, USA)

API intruments (Bio Rad, USA)

Refrigerator (-20°C) (Forma Sciencetifix, USA)

Incubation room 37°C, 43°C (N.I.H.)

Refrigerator centrifuge (4°C) (Sigma, USA)

Heat Block 100°C (Thermolyne)

Anaerobic glove box (Forma scientific)

Anaerobic jar (Oxoid, BBL)

Laminar air flow (Air tech)

pH meter (Autocal)

vortex mixer (Chiltern)

Thermal cycles (Bio Rad, USA)

Power supply (Bio Rad, USA)

Water bath (Bio Rad, USA)

Heat block (Scientific, USA)

Centrifuge (Tomy Seiko, Japan)

pH meter (Orion, USA)

Microcentrifuge (Tomy Seiko, Japan)

Magnetic stirrer (VELP Scientifica, Italy)

Automated thermal cycle (perkin-Elmer Cetus, USA)

Electrophoresis chamber (Bio Rad, USA)

Power supply (Bio Rad, USA)

UV transilluminator (Apectroline, USA)

Water bath shaker (Yamato, Japan)

Polaroid camera (Polaroid, USA)

Colling water bath

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Biological satety cabinet (Yamato, Japan)

Automatic pipette (Brand, Germany)

Rotary shaker (Bellco Glass, USA)

Incubator (Sanyo, Japan)

Refrigerator (Sharp, Japan)

Freezer-20°C (Sanyo, Japan)

Balance (Mettler, Japan)

Autoclave (Yamato, Japan)

Spectrophotometer (Bio-Tek instruments, USA)

Anaerobic jar (Oxioid, England)

Microwave (Sanyo, Japan)

Vortex mixer (Scientific, USA)

Contour-clamped homogenous electric field apparatus (CHEF-DR II system), (Bio Rad, USA)

Enzyme and molecular marker

Sma I (Bio-Labs, New England)

λ ladder marker (Bio Rad, USA)

Lysozyme (Sigma, USA)

Proteinase K (Sigma, USA)

Taq polymerase (Promega, USA)

APPENDIX II

Reagents

1. TES buffer
   - 50 mM Tris base (pH 8.0)
   - 5 mM EDTA (pH 8.0)
   - 50 mM NaCl

Tris-base, EDTA and NaCl were dissolved in ultra pure water and then sterilied by autoclaving at 121°C, 15 pounds pressure, for 15 minutes. The buffer was stored at rood temperature.

2. 25% sucrose in TES

- Sucrose	10	g
- TES	40	ml

Sucrose was dissolved in TES and sterilize in the autoclave at 115°C, 15 pounds pressure, for 10 minutes.

3. Lysis buffer #1

- Lysozyme	125	mg
- 25% sucrose in TES	25	ml

Lysozyme was dissolved in TES. Aliquot 5 ml of mixture to each of five 5 ml tubes. The buffer was stored at -20°C until used.

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4. TEN buffer

 - 10 mM Tris base (pH 7.5)
 - 1 mM EDTA (pH 8.0)
 - 10 mM Nacl

Tris-base, EDTA and NaCl were dissolved in ultra pure water and then sterilied by autoclaving at 121°C, 15 pounds pressure, for 15 minutes. The buffer was stored at room temperature.

5. Lysis buffer #2

- 10% Sarkosyl	16	ml
- 5 mg/ml proteinase k	4	ml
- TEN	100	ml

Combine all the ingradients and was stored at -20°C untill used.

6. 0.5 M Tris-HCI (pH 8.0)

- Tris-base	60.55	g
- Distilled water	1000	ml
- HCl (Concentrate)		

Tris-base was dissolved in 700 ml distilled water. Add HCI until pH to 8.0. Add distilled water (Deionized) to increase the volumn to 1000 ml.

7. 0.5 MEPTA pH 8.0

- EDTA 93.05 g
- Distilled water 350 ml
- NaOH pellets

EDTA was dissolved in distilled water and adjust the pH to 8.0 with NaOH pellets

8. 1XTE buffer

- 0.5 M Tris
- 0.5 MEDTA

Tris-base and EDTA were dissolved in distilled water and then steriled by autoclave at 121°C, 15 pounds pressure, for 15 minutes. The buffer was stored at 4°C until used.

9. Lysis buffer

- 10 mM Tris-HCI (pH 7.6)
- 10 mM EDTA
- 1 mg/ml Mutanolysin

Combine tris-HCI and EDTA and then autoclave. The buffer was stored at room temperature and add mutanolysin when the buffer was used.

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10. 5X TBE

- Tris base 54.0 g
- boric acid 27.5 g
- 0.5 EDTA pH 8.0 20 ml
- Distilled water 1000 ml

11. 1.6% agarose, low melting

- agarose 0.16 g
- TES 10 ml

12. Lysis Solution I

- 1% SDS 0.06 g
- 0.5 M EDTA 6.0 ml
- 20 mg/ml Protease 4.8 ml

13. Lysis Solution II

- 1% Sarcosyl 0.06 g
- 0.5 M EDTA 5.4 ml
- 20 mg/ml Protease 0.6 ml

Mixed 1% sarcosyl and EDTA. ADD Protease When the buffer was used.

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14. PMSF + 1xTE (1 : 10)

- PMSF (phenylethyl sulfonyl fluoride)	2	ml
- 1 XTE	18	ml

Combine PMSF with TE and was stored at -20°C.

15. Ethidium bromide solution

Ethidium bromide stock solution (5 mg/ml in water) was diluted to 0.5 μg/ml in water.

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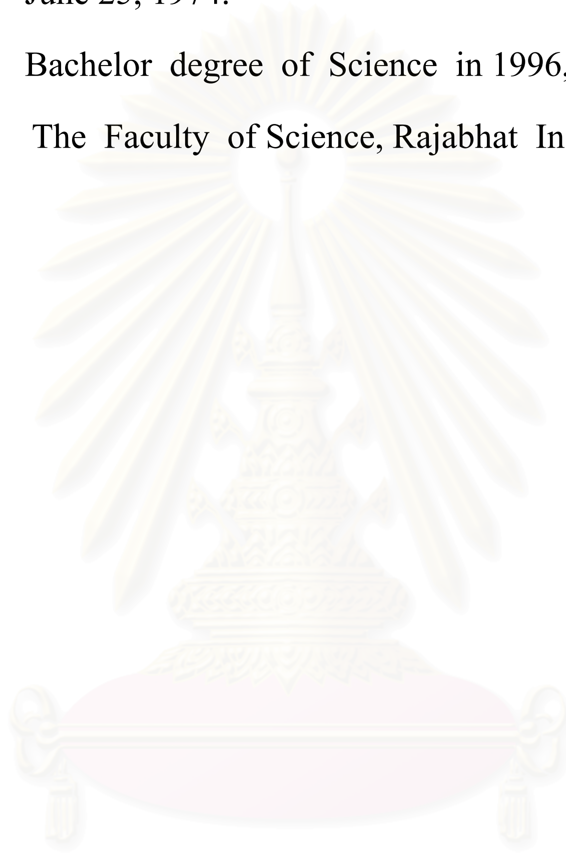

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