IDENTIFICATION OF SEROTYPE SPECIFIC DNA MARKER FOR *SALMONELLA* TYPHIMURIUM BY RAPD-PCR METHOD

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CEREN AKSOY

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Approval of the Graduate School of Natural and Applied Sciences

_____

Prof. Dr. Canan Özgen

Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science

_____

Assoc. Prof. Dr. Dilek Sanin

Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

_____   _____

Prof. Dr. Ufuk Bakır   Assoc. Prof. Dr. G. Candan Gürakan

Co-Supervisor   Supervisor

Examining Committee Members

Prof. Dr. Haluk Hamamcı   (METU, FDE)   _____

Assoc. Prof. Dr. G. Candan Gürakan   (METU, FDE)   _____

Prof. Dr. Zümrüt B. Ögel   (METU, FDE)   _____

Assist. Prof. Dr. Dilek Keskin   (METU, ES)   _____

Dr. Remziye Yılmaz   (Tarım Bak.)   _____

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Name, Last name : Ceren Aksoy

Signature :

ABSTRACT

IDENTIFICATION OF SEROTYPE SPECIFIC DNA MARKER FOR
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Aksoy, Ceren

M. Sc., Department of Biotechnology

Supervisor: Assoc. Prof. Dr. G. Candan Gürakan

Co-Supervisor: Prof. Dr. Ufuk Bakır

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This study was performed for the identification of specific DNA marker using RAPD-PCR (Random Amplified Polymorphic DNA) method for serotype *Salmonella* Typhimurium which is one of the most prevalent serotype causing food poisining all over the world.

The Primer 3 (RAPD 9.1), 5'-CGT GCA CGC-3', was used in RAPD-PCR with 35 different *Salmonella* isolates. 12 of them were serotype *Salmonella* Typhimurium and 23 of them were belonging to other six different serotypes.

Accordingly, two different 300 bp and 700 bp sized amplification products were obtained from the 12 different *Salmonella* Typhimurium isolates. On the other hand, other 23 *Salmonella* isolates of six different

serotypes gave only 300 bp amplification band while 700 bp amplification band was not observed with Primer 3 (RAPD 9.1).

After the discovery of 700 bp fragmentwhich was specific for S. Typhimurium, it was decided to sequence. The 700 bp band was ligated onto the vector pUC 19 to sequence. The cloninig operation gave positive results by the formation of blue and white colonies, but plasmid isolation process from white colonies containing the ligated vector was not achieved. Therefore, sequencing of the 700 bp fragment together with plasmid DNA could not be completed. However it wil be sent to USA for sequencing.

According to these results, it was discovered that 700 bp amplification product was found as a specific polymorphic region for *Salmonella* Typhimurium after RAPD application on genomic DNA and this band can be used as a specific marker for detection and identification of *Salmonella* Typhimurium.

Keywords: *Salmonella* Typhimurium, RAPD-PCR, DNA marker, identification.

ÖZ

SALMONELLA TYPHİMURİUM İÇİN SEROTİP SPESİFİK DNA İŞARETLEYİCİSİNİN RAPD-PCR METODU İLE TANISI

Aksoy, Ceren

Yüksek Lisans, Biyoteknoloji Bölümü

Tez Danışmanı: Doç. Dr. G. Candan Gürakan

Yardımcı Tez Danışmanı: Prof. Dr. Ufuk Bakır

Eylül 2004, 80 sayfa

Bu çalışmada tüm dünyada yaygın olarak gıda zehirlenmelerine neden olan *Salmonella* Typhimurium serotipi için spesifik bir DNA işaretleyicisinin RAPD-PCR tekniği ile tespit edilmesi amaçlanmıştır.

Çalışmada Primer 3 (RAPD 9.1), 5'-CGT GCA CGC-3', kullanılarak 12 tanesi serotip *Salmonella* Typhimurium, 23 tanesi de farklı altı *Salmonella* serotipine ait olmak üzere, 35 farklı *Salmonella* isolatı için RAPD-PCR yapılmışır.

Buna göre serotip *Salmonella* Typhimurium için Primer 3 (RAPD 9.1) ile 300 bp ve 700 bp olmak üzere iki farklı amplifikasyon ürünü elde

edilmiştir. Diğer yandan altı faklı *Salmonella* serotipine ait 23 isolat aynı primer ile sadece 300 bp'lik bant verirken, 700 bp'lik amplifikasyon bandı hiçbir isolat ile elde edilememiştir.

700 bp'lik fragmantın Salmonella Typhimurium için spesifik olduğu bulunduktan sonra sekanslanmasına karar verilmiştir. Sekanslamak amacıyla 700 bp'lik bant vektör pUC 19 üzerine takılmıştır. Klonlama işlemi mavi ve beyaz koloniler oluşumu şeklinde pozitif sonuçlar verirken, klonlanmış vektörü taşıdığı düşünülen beyaz kolonilerden plasmid isolasyonu başarılamamıştır. Bu nedenle, 700 bp'lik bandın plasmid DNA'sı ile birlikte sekanslanması işlemi gerçekleştirilememiştir.

Bu sonuçlara dayanarak 700 bp'lik amplifikasyon ürünü *Salmonella* Typhimurium serotipi için spesifik, polymorfik bir bölge olarak bulunmuş ve bu bandın *Salmonella* Typhimurium serotipinin teşhis ve tanımlanmasında spesifik bir marker olarak kullanılabileceği kanısına varılmıştır.

Anahtar kelimeler: *Salmonella* Typhimurium, RAPD-PCR, DNA işaretleyicisi, tanı.

To My Father

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INTRODUCTION

1.1 Genus Salmonellae

Salmonellae are members of Enterobacteriaceae. They are Gram-negative, non-sporforming rods (typically $0,5\mu m$ by $1\text{-}3\mu m$) which are facultatively anaerobic, catalase-positive, oxidase-negative, and are generally motile with peritrichous flagella. They utilize citrate as a sole carbon source and generally ferment glucose but not sucrose or lactose.

Growth has been recorded temperatures just above 5°C up to 47°C with an optimum at 37°C. Salmonellae are heat sensitive and are readily destroyed by pasteurization tempratures. In frozen foods numbers of viable *Salmonella* decline slowly, the rate decreasing as the storage temprature decreases. The minimum a_w (water activity) for growth is around 0,93 but cells survive well in dried foods, the survival rate increasing with the a_w reduced. The minimum pH for growth varies with the acidulant from 5,4 with acetic acid to 4,5 with hydrochloric and citric acids. Optimal growth occurs around pH 7 (Adams, 1995).

There are several problems and a lot of proposal in the nomenclature of genus *Salmonella.* According to the Bergey's Manual (1984) the genus *Salmonella* contain a single species S. *choleraesuis* (now known as S. *enterica*) which comprises five subspecies on the basis of DNA-DNA hybridization. These subspecies are; *Salmonella enterica* subspecies enterica,

Salmonella enterica subspecies salame, *Salmonella enterica* subspecies arizonae, *Salmonella enterica* subspecies indica, *Salmonella enterica* subspecies houtenae (Jay, 1992). However, Le Minor *et al.* (1982-1986) proposed the name *Salmonella choleraesuis* for the single *Salmonella* species with following seven subspecies: *Salmonella choleraesuis* subspeceis *arizonae*, *Salmonella choleraesuis* subspeceis *bongori*, *Salmonella choleraesuis* subspeceis *choleraesuis*, *Salmonella choleraesuis* subspeceis *diarizonae*, *Salmonella choleraesuis* subspeceis *houtenae*, *Salmonella choleraesuis* subspeceis *indica*, *Salmonella choleraesuis* subspeceis *salame*. In 1989, Reeves *et al.* elevate the supspecies *Salmonella choleraesuis* subspecies *bongori* in rank to species. Consequently, the species *Salmonella choleraesuis* (now known as *Salmonella enterica*) only encompasses six subspecies, which are enterica, salame, arizonae, diarizonae, indica, and houtenae, also known as subspecies I, II, IIIa, IIIb, IV, and VI, respectively.

The taxonomic classification of *Salmonella* has been continually revised over the years. Beyond the level of subspecies, serotyping is used for the differentiation, and serotypes have been described within *S. enterica* subspecies *enterica* on the basis of somatic (O) antigens, flagellar (H) antigens and capsular (Vi) antigens (Uzzau *et al.*, 2000). The classification of organisms by antigenic analysis is based on the Kauffman and White scheme. Classification by this scheme makes use of both somatic O and flagellar H antigens. The somatic O antigen is essential for serological typing. *Salmonella enterica* subspecies *enterica* consist of around 2,300 serotypes based on the 67 different O-antigen groups which are designated A, B, C, and so on, for further classification, the flagellar H antigens are employed. H antigens are two types: specific phase or phase 1, and group phase or phase 2. Phase 1 antigens are shared with only a few other species or varieties of *Salmonella*; phase 2 may be more widely distributed among several species (Jay, 1992). Serotypes are identified by antigenic formulae, taking the general form O antigen, phase 1 and phase 2 antigens, in which O antigens are designated by numerals, the phase 1 antigens by small letters, and phase 2 antigens by arabic numerals.

Thus , the complete antigenic analysis of *S. enterica* is as follows: 6, 7, c, 1, 1, 5, where 6 and 7 refer to O antigens, c refer to phase 1 flagellar antigens, and 1 and 5 refer to phase 2 flagellar antigens. *Salmonella* subgroups of this type are refered to as serovars.

Table 1.1 Antigenic Structure of Some *Salmonella* Serotypes

| Group | Specific/Serovars | O Antigens | H antigens | |
			Phase 1	Phase 2
A	*S*. Paratyphi A	1, 2, 12	a	(1,5)
B	*S*. Schottmuelleri	1, 4, (5), 12	b	1, 2
	S. Typhimurium	1, 4, (5), 12	i	1, 2
C1	*S*. Hirschfeldii	6, 7, (Vi)	c	1, 5
	S. Oranienburg	6,7	m, t	-
	S. Montevideo	6,7	g, m,s,(p)	(1,2,7)
C2	*S*. Newport	6,8	e, h	1,2
D	*S*. Typhi	9, 12, (Vi)	d	-
	S. Enteritidis	1, 9, 12	g, m	(1,7)
	S. Gallinarum	1, 9, 12	-	-
E1	*S*. Anatum	3,10	e, h	1, 6

Name of many of these thousand of serotypes have been often written for convenience as if they were (but they really are not!) species names in addition to type species *Salmonella enterica,* such as *Salmonella enteritidis, Salmonella typhi, Salmonella typhimurium and Salmonella arizonae.* The present convention is to capitalize serovar designation and indicate it (without italicizing it) after the genus, species or subspecies name; for example, *Salmonella typhimurium* would now be written *Salmonella* Typhimurium, *Salmonella enterica* serovar Typhimurium, or the very cumbersome

Salmonella enterica subsp. *enterica* serovar Typhimurium. (Lindquist *et al.*, 1998).

Salmonella serotypes are normally divided into two groups on the basis of host range; host adapted and host restricted (ubiquitous). The first group of host adapted serotypes; such as *Salmonella* Typhi, *Salmonella* Paratypi A, *Salmonella* Paratyphi C only infects humans, the second group of host adapted serotypes; such as *Salmonella* Dublin and *Salmonella* Gallinarum can cause disease in more than one host species. Host restricted serotypes, for example *Salmonella* Typhimurium and *Salmonella* Enteretidis, are pathogenic both humans and animals (Uzzau *et al.*, 2000).

1.2 Distiribution of *Salmonella* and Salmonellosis

Salmonella is one of the most important pathogens involved in human foodborne illnesses in the developed world and the majority of cases of human salmonellosis are due to the consumption of contaminated egg, poultry, pork, beef, and milk products (Cerro *et al.*, 2002).

The primary habitat of Salmonellae is the intestinal tract of animals such as birds, reptiles, farm animals, and humans. Although their primary habitat is the intestinal tract, they may be found in other parts of the body from time to time. As intestinal forms, the organism are excreted in feces from which they may be transmitted by insects or other living creatures to a large number of places. As intestinal forms, they may also be found in water, especially polluted water. When polluted water and foods that have been contaminated by insects or by other means are consumed by humans and other animals, these organisms are once again shed through fecal matter with a continuation of the cycle. The augmentation of this cycle through the international shipment of animal products and feeds is in the large part responsible for the worldwide distribution of salmonellosis and its consequent problems (Jay, 1992).

Food animals harbour a wide range of *Salmonella* serotypes and so act as source of contamination, which is of paramount epidemiological importance in non-typhoid human salmonellosis. The process of removing the gastrointestinal tract during slaughtering of food animals is regarded as one of the most important source of carcass and organ contamination with *Salmonella* at abattoirs. Food items such as poultry, meat and meat products are the common source of foodborne salmonellosis. Contamination of meat by *Salmonella* may occur at abattoirs from the excretion of symptomless animals, contaminated abattoir equipment, floors and personnel and the pathogen can gain access to meat at any stage during butchering. Cross contamination of carcasses and meat products could continue during subsequent handling, processing, preparation and distribution (Molla *et al.*,2003).

The genus Salmonellae is of great concern to the food industry. Processed foods are not permitted to contain any *Salmonella* cells. The reason for this 'zero tolerance' is that salmonellae are responsible for severe, acute gastroenteritis. The genus Salmonellae are responsible for a major portion of the cases of gastroenteritis each year in the United States. The Food Safety and Inspection Service (FSIS) inspects meat, poultry, and egg products to ensure they are safe, wholesome, and accurately labeled. FSIS is amending its regulations to clarify and strengthen enforcement of its zero-tolerance policy regarding visible fecal material on poultry carcasses. The final rule formalizes what has been a long-standing policy of the Agency. The zero tolerance policy for visible fecal contamination is an important food safety standard because fecal contamination is a major vehicle for spreading disease-causing microorganisms, such as *Salmonella*, to raw poultry. FSIS expects that the final rule, together with other food safety initiatives most notably the Pathogen Reduction/Hazard Analysis and Critical Control Point (HACCP) regulations-- will help to improve the safety of raw poultry products and bring about declines in foodborne illness attributable to poultry consumption. Because the zero fecal tolerance for poultry parallels an already existing zero fecal tolerance for meat, the rule also improves consistency between the regulations

governing meat and poultry products. Other activities FSIS has undertaken to improve consistency include permitting steam pasteurization as an alternative to knife trimming for beef.

Non-typhoidal Salmonellae are among the main causes of food-borne illness especially in the 'western world'. *Salmonella* Typhimurium is a leading cause of of non-typhoid salmonellosis. *Salmonella* Typhimurium strain LT2, the principal strain for cellular and molecular biology in *Salmonella*, was isolated in the 1940s. The chromosome of *Salmonella* Typhimurium LT2 is 4,857 kb and it has a 94 kb virulence plasmid, and full genome of S. Typhimurium LT2 was sequenced (McClelland *et al.,* 2001).

In France for many years, *Salmonella enterica* subspecies enterica serotype Typhimurium has been the commenest serotype isolated from human cases, although from 1990-1994 it was overtaken by *Salmonella enterica* serotype Enteritidis. *Salmonella* Typhimurium is the commenest *Salmonella* infection in cattle and is the most frequent serotype among *Salmonella* isolates from beef products as well as pork and pork meat products. Several outbreaks of *Salmonella* Typhimurium food poisininig associated with eating beef have been reported. Pork and pork meat products have been implicated in outbreaks in England, Wales; and Italy as well as in a case-control study in Britain. (Delarocque-Astagneau *et al*., 2000) . According to the survey was carried out in Taiwan, *Salmonella* Typhimurium is the most common serotype. *Salmonella* isolates obtained from patients and also common bacterial contaminant of foods such as milk and chicken meat (Lın J.S. *et al*., 1999).

The results of investigations indicate that outbreaks of salmonellosis also was caused by milk and milk products including fresh milk, fermented milks, ice cream, dried milk powder and raw milk cheese. In adition to the milk, eggs may carry the *Salmonella* , foods made with such eggs if not cooked or pasteurized properly may carry live organism (Nickerson and Sinskey, 1972). In the United States, salmonellosis accounts for about 60% of all bacterial disease outbreaks confirmed cases were reported in 1989. The actual number of

cases, however, is estimated to be close to 2 million. Approximately 5 million analytical tests are performed annually in the United States , thereby making tests for *Salmonella* detection among the most lucrative in the diagnostics markets (Feng *et al*., 1992).

There are three syndromes observed following the consumption of *Salmonella* as salmonellosis, typhoid fever, and paratyphoid fever. Incubation period of salmonellosis is especially between 5-72 hours and diarrhea, abdominal pain, chills, fever, vomiting, dehydration, prostration, anorexia, headache observered during this period. Salmonellosis can be caused by *S*. Enteritidis*, S*. Ttyphimurium*, S*. Heilderberg*, S*. Derby*, S*. Infantis*, S*. Montevideo. Typhoid fever which is caused by *S*. Typhi continue between 7-28 days and septicemia and malaise, high fever, caugh, nausea, dierrhea, constipation, slow pulse rate, tender, enlarged spleen, bleeding from bowel is observed during this period. Etiologic agents of paratyphoid fever are; *S*. Enteritidis cause bloodstream between 1-15 days, *S*. Paratyphi A and *S*. Paratyphi B cause headache, fever, profuse perspiration, nausea*, S*. Paratyphi C *and S*. Sendai cause vomiting, abdominal pain, diarrhea during 1-3 weeks (Fraizer , 1988).

Salmonella infections can be prevented following measures such as adequete cooking of foods from animal sources, suitable refrigiration, protection of food from contamination, periodic analysis of stool samples from food handlers, and good personel sanitary and hygienic practices. Since infections with *Salmonella* are a world wide health problem, detection and identification of *Salmonella* is very important.

1.3 Detection, Identification, and Characterization of Salmonellae

The analysis of multiple bacterial isolates by phenotypic or genotypic methods can be used to identify characteristics within a particular species. Due to the problems in the use of phenotype based metods which are increasingly challanged by the use of DNA-based methods. In the last few years innovative molecular techniques have provided powerful tools for direct DNA analysis

which offers many advantages. One of the more important advantages is that since DNA can always be extracted from bacteria, all bacteria should be typeable. Another is that the discriminatory power of DNA-based methods is greater than that of phenotypic procedures (Bush *et al*., 1999). A listing of some of the charactheristics of bacterial typing systems can be seen in Table 1.2.

An ideal typing system (Hawkey, 1989) should: (a) be able to type the vast majority of strains encountered; (b) have good discrimination with the ability to recognize a reasonable number of types; (c) show goood reproduciblity over a long period of time and in different centers; (d) be readily applicable to natural isolates as opposed to laboratory collections of strains; (e) should not be too complicated or expensive.

Because Salmonellae cause major food poisining cases and outbreaks in the world, detection, identification and also molecular characterization of *Salmonella* is very important for human and animal health and food industry. For these reasons Salmonellae strains isolated from patients of food poisining cases and suspected animals are used in detection, identification and molecular characterization studies   both with phenotypic and DNA-based genotypic methods.

Table 1.2 Charactheristic of Bacterial Typing Systems

Typing System	Proportion of Strain Type	Reproducibility	Discriminatory Power	Ease of Interpretation	Ease of Performance
I.Phenotypic Methods					
Biotyping	All	Poor to fair	Poor	Excellent	Excellent
Serotyping	Most	Good	Fair	Good to excellent	Fair to good
Antibiotic Susceptibility	All	Fair	Poor	Excellent	Very good to excellent
Phage Typing	Variable	Fair	Fair	Fair to good	Poor to fair
II. Genotyping Methods					
Plasmid Fingerprinting	Variable	Fair to good	Good	Good	
Restriction Endonuclease Analysis	All	Very good	Good	Poor	Excellent
Ribotyping	All	Excellent	Fair to good	Very good to excellent	Excellent
Pulsed-Field Gel Electrophoresis	All	Excellent	Excellent	excellent	Fair to good
PCR Ribotyping	All	Very good	Good	Excellent	Fair to good
PCR-RFLP	All	Excellent	Good	Excellent	Very good to excellent
RAPD-PCR	All	Good	Very good to excellent	Very good	Very good to excellent

1.3.1 Phenotypic Methods

Phenotypic methods are those that characterize the products of gene expression in order to differentiate strains. Properties such as biochemical profiles, bacteriophage types, antigen present on the cell's surface, and antimicrobial susceptibility profiles all are examples of phenotypic properties that can be determined in the laboratory, methods such as biotyping, serotyping, phage-typing, antibiotic susceptibility, immunologic methods etc. (Tenover *et al*., 1997).

There are problems with many of these methods is that because of the need for specialized reagents, only a few reference labs around the world can realibly carry them out. Other problems with some phenotypic methods include nontypeability of strains, nonreproducibility, and lack of discriminatory power (Farber *et al*., 1996).

1.3.1.1 Biotyping

Frequently, initial differentiation within a newly-delineated species is achived by examining the cultural and biochemical characteristics of a large collection of individual strains belonging to the species. Such charactheristics may include colonial morphology, growth requirements, fermentation ability, carbon source utilization and antibiotic resistance.

In theory such properties are easy to determine, but in practice the determination of biotypes may not always be straightforward. Differences in colonial morphology are often extremely subtle and, therefore, can be rather subjective (Towner, 1993) that is the discriminatory power of bioyping is low. Biotyping, like most phenotypic methods, has only modest reoroducebility, because microorganisms can alter unpreditably the expression of many cellular products (Tenover *et al*., 1997).

Conventional culture method is still most commonly used biotyping method for the detection of *Salmonella* in foods or clinical samples although it is labor intensive and time consuming, requiring 4 to 7 days. These methods includes preenrichment, selective broth enrichment, selective and differential plating, biochemical characterization of suspected isolates, and serological confirmation of *Salmonella* serotypes. Preenrichment is carried out for the recovery and growth of cells, then to inhibit competitive bacteria selective broth enrichment is performed (Lin *et al*., 1999).

In one study 25 g of neck skin samples were aseptically trimmed and homogenised for 1 min in 225 ml aliquots of buffered peptone water using stomacher. Following overnight incubation at 37°C, 0,1 ml aliquots were inoculated in tubes containing 10 ml Rappaport-Vassiliadis (RV) broth and incubated for 48 hour 42°C as previously recommended. Brilliant Gren Agar (BGA) plates were inoculated from each of the RV broths after 24 and 48 hour and incubated for 18-24 h at 37°C as previously described. Suspect colonies were confirmed biochemically by inoculating into lysine decarboxylase broth, urea broth and triple sugar iron agar slopes with final confirmation carried out using specific *Salmonella* O and H agglutinating antisera (Whyte *et al*., 2002).

1.3.1.2 Serotyping

Serotyping is one of the oldest typing procedures, but particularly when used in conjuction with other typing methods, still represents an important tool for routine identification and typing of many microbial species. Based on reactions with specific antisera (e.g. antisomatic, antiflagellar or anticapsular), raised according to the antigenic structure of microbes in each group, the method has been developed in detailed for bacteria belonging to the Enterobacteriaceae such *Salmonella* and *Shigella* (Ewing, 1986).

Serotyping of *Salmonella* is the most common method used to differentiate strains, which are epidemiologically the smallest bacterial units

from which isolates share the same phenotypic and genotypic traits. Serotyping separates strains based on their somatic (O), capsular (Vi), and flagellar (H) antigens into distinct serotypes. Classically, serotyping was used to divide *Salmonella* into distinct species based on their serotypes, giving rise to one species per serotype. Of the two species of *Salmonella, S. enterica* and *S. bongori,* over 99% of serotypes are grouped into the species *S. enterica,* and nearly 60% of them belong to the subspecies *enterica.*

To perform serotyping, a suspension of a *Salmonella* isolate is mixed and incubated with a panel of antisera that recognize specific O and H epitopes. The agglutination profiles generated are used to determine the particular serotype of the isolate being tested. A comprehensive serotyping scheme used to determine the particular serotype is the Kauffmann-White identification system, which has been extensively used by many public health organizations. Because of its wide acceptance as a method to differentiate *Salmonella* strains, serotyping is an important tool in public health. However, maintaining stocks of typing sera (including the >2200 antisera reqired for definitive Salmonella typing) is a major limitation of this method. That is the main dissadvantages seem to be associated with problems in antisera production and standardization of methodology (Yan *et al.*,2003).

1.3.1.3 Phage Typing

Bacteriophages (phages) are viruses capable of infecting bacteria, leading in some cases to lysis of bacterial cell and release of further infective phage particles, while in other cases the phage can persist as a relatively stable prophage within the bacterial cell. Phage typing is a method for bacterial strain identification that is based upon sensitivity to defined collections of bacteriophages which have been selected to provide the maximum sensitivity for differentiating strains within a particular species. Typing may be 'direct', i.e. based on direct sensitivity to either unadapted or adapted phages, or

'indirect', i.e. based on detection and identification of phages present as prophages in bacteria.

Phage typing remains the major typing method for *Staphylococcus aureus*, and is also particularly useful for subdividing serotypes of *Pseudomanas aeruginosa* and *Salmonella/Shigella* spp. (Towner, 1993). Different bacteriophages are able to selectively infect *Salmonella* isolates due to differences in the phage and phage receptor present on the surface of the bacterium (Snydler, 1997). Phage typing has been used to describe pandemic clones of Salmonella, such as *S. enterica* serotype Typhimurium definitive type 104 (DT 104) that causes severe gastrointestinal illness and is typically resistant to multiple antimicrobials (Randall *et al*., 2001).

It can be concluded that phage typing is a higly sensitive, but technically demanding method of typing that requires the maintenance of multiple biologically active phage stocks, it is usually performed only by public health and reference laboratories.

1.3.1.4 Antibiotic Susceptibility

The antimicrobial susceptibility (antibiogram) is analyzed by the growth of an isolate in the presence of given antibiotic. Antimicrobial susceptibiliy patterns also have relatively poor discriminatory power, because antimicrobial resistance is under tremendous selective pressure in healthcare institutions and often is associated with mobile genetic elements (eg. transposons and plasmids). Changes in antibiograms also may reflect spontaneous point mutations, such as seen with fluoroquinolones. Thus, isolates that are epidemiologically related and otherwise genetically indistingushable may manifest different antimicrobial susceptibilities due to acquisition of new genetic material over time or the loss of plasmids. Conversly, unrelated isolates may have indistingushable resistance profiles, which may represent acquisition of the same plasmid by multiple species (Tenover *et al*., 1997).

1.3.1.5 Immunological Methods

1.3.1.5.1 Immunoblotting

Immunoblotting is used for strain differantiation of a variety of medically important microorganisms. This technique involves the electrophoretic transfer of proteins or LPS molecules which are separated by SDS-PAGE from polyacrilamid nitrocellulose or nylon membrane. Membranes are then incubated with a polyclonal serum or monoclonal antibody, after which antibody binding to antigens is detected by incubation with tagged antibody conjugate and an appropriate detection system using radiolabels. After development of blots to visualize the antigenic profile, membranes are washed and dried. They are then photographed to provide a permanent record.

Immunoblotting has been employed to investigate the validity of serogroups of some Gram-negative bacteria estabilished with conventional typing sera. For example, LPS from isolates of *Pasteyrella haemolytica* assigned originally to different serotypes showed very similar immunoblot profiles, supporting results of earlier studies which suggested that the assigned serotypes were artefactual (Rimler *et al*., 1984).

Immunoblotting are not ideal method for some applications for antigenic analysis of microorganisms. In particular, some epitopes on protein antigens that may be of potential use for typing may be destroyed during sample preparation for SDS-PAGE. This method also have some technical disadvantages which are assotiated with poor transfer and distortion of profiles because of trapping of air bubbles within the blotting sandwich, or because of incomplete equilibriation of the gel with the transfer buffer before blotting (Towner, 1993).

1.3.1.5.2 Enzyme-Linked Immunosorbent Assay (ELISA)

Since the analysis of large numbers of strains and antibodies by immunoblotting have some problems, ELISA techniques can be used to overcome some of these problems.

Close examination and comparison show that all *Salmonella* ELISAs share many common features. Kit assays require enrichment steps to resuscitate injured cells and to selectively amplified salmonella. The enrichment temperatures and incubation periods vary with the kits are and with the types of food analysed. All ELISA kits are designed in a ''sandwich'' or capture format, i.e., antibody coated polystrene wells are used to capture salmonella antigen, and a second antibody to salmonella conjugated with an enzyme is added to form an antibody-antigen-antibody (sandwich) complex. The sandwich complex is then determined by a colorimetric enzyme substrate, and the results are recorded either visually or with a spectrophotometer (Feng *et al*., 1992). Almost all *Salmonella* ELISA kits use alkaline phosphatase or horseradish peroxidase enzyme conjugates with a colorimetric substrate system. For example, in Tek^{TM} ELISA test system the detector antibodies are conjugated with horseradish peroxidase, which catalyzes the oxidation of tetramethylbanzidine and as a result a blue color develops. The enzymatic reaction is stopped with sulphuric acid which at the same time changes the blue to a yellowcolor. The intensity of the resulting yellow color solution absorbance value is measured at 450 nm with a microelisa reader, that allows the conclude whether there are *Salmonella* antigens in the sample or not (Molla, *et al*., 1996).

1.3.1.6 Rapid Screening Methods

Rapid screening methods would be performed directly on the food product without the need of enrinchment of the sample, but none of the

screening methods are available to detect the low number of *Salmonella* in foods.

EF-18 agar which is a new selective and differential culture medium for *Salmonella*, was developed by Entis *et al*. (1991) to use with the hydrophobic grid membrane fitler (HGMF). The HGMF/EF-18 agar method was evaluated against the conventional culture method (AOAC/BAM) using a total of 954 samples comprising 25 product categories. The HGMF/EF-18 method detected 653 *Salmonella* positive samples and AOAC/BAM method detected 654. The HGMF/EF-18 method with an overall false negative rate of 2% was determined to be equivalent in selectivity to AOAC/BAM procedure. The presumptive false positive rates were 0.3% for the HGMF/EF-18 and 7.95 for AOAC/BAM (Ören, 1997).

1.3.2 Genotypic Methods

Genotyping which involves direct DNA-based analysis of chromosomal or extrachromosomal (e.g., plasmid) genetic material, has many advantages over traditional typing procedures. The major advantage lies in its increased discriminatory power, i.e., in its ability to distinguish between two closely related strains. In examining the discriminatory ability of a particular method, it is useful to otherwise identical, when tested by other techniques. Other advantages of genotyping include: (i) DNA can always be extracted from bacteria so that all starins are typeable. The only exception here would be in the case of plasmid typing, which will be discussed later on; (ii) analytical strategies for the genotypic methods are similar and can be applied to DNA of any source; (iii) in general, genomic DNA is a stable characteristic and its composition is independent of cultural conditions or methods of preperation; and (iv) it allows for statistical data analysis and is amenable to automation (Arbeit *et al*., 1995, Bingen *et al*., 1994). Genotypic methods classified as restriction enzyme based methods and PCR based methods.

1.3.2.1 Restriction Digestion Based Methods

1.3.2.1.1 Chromosomal DNA Restriction Profiles

For this type of analysis, chromosomal DNA is digested by restriction endonucleases and the generated fragments are separated by agarose gel electrophoresis. Restriction endonucleases are enzymes cutting DNA at a defined position within (or close to) a specific recognition sequence. Since restriction endonucleases are highly specific, complete digestion of a given DNA provides reproducible pattern of DNA fragments whereby the number and size of fragments depend on the DNA composition. Variation in the pattern of fragments is called restriction fragment length polymorphism (RFLP) and can result from a minor change of DNA e.g., single base substitution within the restriction enzyme recognition and/or cleavage site, or major changes like insertions, deletions or sequence rearrangements. A limitation of restriction enzyme digestion of whole chromosomes is the generation of a large number of as different fragments. The separation by conventional gel electrophoresis leads to a smear-like appearance of the DNA on the gel and the yielded band pattern are also too complex to be properly compared. To master this problem, the method was combined with nucleic acid probes (Busch *et al*, 1999).

1.3.2.1.2 Ribotyping

The use of rRNA-based probes for typing purposes is refered as 'ribotyping'. Many of the rRNA gene sequences found in the bacteria appears to have changed little during evolution, that means rRNA genes are very higly conserved. Therefore, probes specific for these sequences can detect a wide range of bacteria with similar rRNA sequences. rRNA genes are present in several copies and organised into operons, which contains individual genes coding for 16S, 23S and 5S rRNA. These individual genes are separated by non-coding spacer regions (Woese *et al.*, 1987).

In practise the steps of ribotyping can be arranged in that manner. Firstly, total DNA is isolated from cultered cells. Then usually by using 6bp recognising restriction enzyme DNA is cut into many small fragments which are separated by agarose gel electrophoresis. Subsequently, hybridization can be performed directly in gel or by transfering the fragments to blotting membrane with radioactively labelled probes. Probes targetted to either 16S, 23S or 5S rRNA genes (Towner, 1997).

The study was performed to ascertain the epidemic genetic types of *Salmonella* Ohio that have been circulating and causing human salmonellosis in Spain during the epidmiological alert of 1998 by Soto et al. (2000). 50 isolates of *Salmonella* serotype Ohio were used in ribotyping with *HincII* which was selected to perform riboyping because it detects a high numbers of restriction sites in the rRNA loci and yield a good discriminatory power within several salmonellae serotypes. *HincII* ribotyping differentiated these Ohio isolates into eight (H1-H8) banding profiles. The h-ribotypes included 10 to 20 bands (of sizes 0.9- 6.3 kbp). The two most frequent H-ribotypes (H2 and H4) showed only two mismatching fragments which could derive from a single insertion/deletion, whereas with regard to H1 showed 11 and 7 mismatching fragments, fact that implies genetic changes. Among 11 lineages (L1-L11) the two most ferquent lineages which are L5 and L3 showed a closer relationshipwith one another. That means two Ohio lineages could be considered endemic in Spain.

1.3.2.1.3 Pulse Field Gel Electrophoresis

The technique is capable of separating large DNA molecules in 50kb-12mb range phsically and by using electrical pulse system is called as 'pulsed field gel electrophoresis'.

Intact cells of bacteria are embedded in agarose gel in order to protect the DNA during the subsequent extraction. The agarose gel plugs are then treated

with detergent and enzymes to isolate the DNA. The embedded chromosomal DNA is cut with rare-cutter restriction endonuclease to generate limited number (10 to 20) of high molecular weight restriction fragments which are ranging in size from 10 to 800 kbp. These fragments then are separated by agarose gel electrophoresis. The molecules subjected to the electrical fields applied alternatively in two directions. By switching the electrical field direction (pulses) frequently, the DNA can snake its way further into the gel and be separated on the basis of sizes. That is smaller fragments migrate faster than larger ones and the DNA fragments can be efficiently resolved (Busch *et al.*, 1999).

The resulting electrophoretic patterns are highly specific for strains from a variety of organisms and also provide an opportunity to examine multiple variations throughout the genome of the organism to identify specific strains and accuracy link them with disease outbreaks. PFGE has great value in epidemiological analysis in the differantiation of pathogenic strains, and in the monitoring of their spread among communities. However, there are some disadvantages of current PFGE protocols which are involved time-consuming steps, tedious procedures for the purification of complete genomic DNA trapped in agarose, lengthy restriction enzyme digest, and extended electrophoresis times (Gautom *et al*, 1997).

1.3.2.1.4 Plasmid Fingerprinting

Plasmid fingerprinting was the first molecular method to be used as a bacterial typing tool. Plasmids are double-stranded extrachromosomal DNA elements that are present in most clinical isolates (Tenover *et al.*, 1985).

In the most basic application, plasmids would be isolated from each bacterial isolate and then seperated electrophoretically in an agarose gel to determine their number and size. Plasmids of the same size, however, may actually be different, i.e., their nucleotide sequences may be different. To circumvent this problems, one can treat the plasmid with a restriction enzyme.

If the two plasmids are different, they could be expected to be cut at different places and thus one would end up with various sizes of DNA fragments which would migrate differently on an agarose gel. Disadvantages of this method are; plasmids usually unstable, some organisms contain few or no plasmids and different plasmids can appear to be the same size even if it is relatively quick and easy method. (Wachsmuth *et al.*, 1991)

1.3.2.2 Polymerase Chain Reaction (PCR)-Based Methods

1.3.2.2.1 The Mechanism of Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is an enzymatic amplification of specific nucleic acid sequences. PCR uses a thermostable polymerase to produce multiple copies of specific nucleic acid regions quickly and exponentially, including non coding regions of DNA as well as particular genes. For example, starting with a single copy of a 1kb DNA, 100 ng of the same sequence can be produced a within few hours.

The PCR method offered as an sensitive approach to the detection and identification of specific organisms in a variety of sample types. Potentially, a characteristic DNA sequence from a single virus particul or cell of a particular organism can be amplified to detectable levels within a very short period of time. The procedure can also be used to amplify RNA after cDNA copy had been generated by reverse transcriptase (Towner , 1993).

Each PCR amplification, which is achived with thermostable DNA polymerase, two synthetic oligonucleotide primers and the four standart deoxyribonucleoside triphosphates, is sub-divided into three steps which are repeated in cycles. All PCR cycles begin by denaturing both template and any previously synthesized double stranded product into single strands at 95°C. As the temperature is lowered toward 50-55°C, primers anneal to the opposite strands of the region of template DNA which have been denaturated by heat.

Finally, the DNa polymerase attached itself to the primer-template complex for primer extention at 72°C by using free dNTPs from medium.

The primers for PCR amplification are synthetic oligonucleotides which are 20-30 nucleotides long, and which are designed by reference to DNA sequence databases. Each pair of primers must act in concert and must relatively specific for their binding sites, since if one or both of primers fails to hybridize, there will be no specific amplification products (Towner, 1993).

The process has been improved greatly by the use of a thermostable DNA polymerase (Taq) isolated from *Thermus aquaticus*. The half-life of Taq polymerase in PCR reaction buffer is 130 min at 92,5°C, and 40min at 95°C. Therefore survive in PCR heat-denaturation step, fresh enzyme does not need to be added at each cycle. PCR automation is done by a PCR machine which can sensitively adjust temprature and time as well as heating and cooling rates.

Specific PCR primer pairs can be used to identify genes that are characteristic of a particular species or strain. Various strategies have also been devised by which the power of PCR can be harnessed for the epidemiological typing of microorganisms. These strategies can be arranged as in below.

In a study was performed by Aobo et.al (1993) ST11/ ST15 primer set which was selected from the squence of a cryptic 2.3 kbp chromosomal fragment of *S*. Typhimurium was used for identification of Salmonella among a control group of non-salmonella *Enterobacteriaceae* strains. The specific PCR product of 429 bp was evaluated for 144 of 146 Salmonella strains, while the two serovars 41:Z4,Z23:- and 44:Z4,Z24:- belonging to subspecies IIIa were false-negative (*S.arizonae*). No PCR products were produced from the 86 non-salmonella *Enterobacteriaceae* strains tested.

Another study was performed by Cohen et.al (1996) by designing primers of the fimA gene of *Salmonella typhimurium*. 376 strains of *Salmonella*

isolated from an.imals and humans and 40 non-*Salmonella* strains were tested with the primers. All *Salmonella* strains with the 85 bp fragment and non-*Salmonella* strains did not amplify, and no non-specific products were amplified under the same PCR conditions. Finally amplification results of fimA gene confirmed by southern hybridization. All *Salmonella* strains amplified the PCR fragment hybridized to the probe.

1.3.2.2.2 PCR Riboyping

PCR ribotyping represent a specialized use of specific primers to amplify particular regions of the genome concerned with rRNA and tRNA synthesis. Traditional ribotyping detects RFLPs by probing chromosomal DNA with rRNA. Although this is a powerful typing method with broad applicability for determining the molecular epidemiology of microrganisms, the technical skill and time required for the hybridization analysis are disadvantages which may limit its application. An alternative direct approach, which circumvents these problems, involves the use of PCR to detect polymorphisms in genes or intergenic spacer regions assotiated with rRNA or tRNA.

Most of the bacterial genera contain 2 and 11 rRNA gene copies per bacterial cell, while the intergenic spacer regions between the 16S and 23S rRNA genes encode various tRNAs and contain several direct repeat sequences in non-coding regions of the gene clusters. While tRNA genes themselves are highly conserved, the lengths of tRNA intergenic spacers are known to vary considerably, e.g. from 2 to 35 bp in *Bacillus subtilis* and 2 to 208 bp in *E. coli.* The tandem arrangement of the tRNA gene clusters allows them to be explored for interspecific intergenic length polymorphism with low stringency PCR employing primers derived from tRNA gene sequences. Using consensus sequences tRNA gene primers it should be possible to generate suitable fingerprints from the majority of microorganisms. The banding patterns produced following PCR amplification of these RNA regions seem to be

reproducible, stable and easily detectable. However, the discriminatory power of PCR-Ribotyping is lower than RAPD typing (Towner, 1997).

In the study of Lagatolla *et al*. (1996), 218 strains of *Salmonella* belonging to 10 different serotypes which are *S. enteritidis, S. londo. S. anatum, S. panama, S. heidelberg, S. agona, S. goldcoast, S.typhimurium. S.infantis, and S. derby* isolated from four Italian hospitals were characterized by PCR-ribotyping. Electrophoretic analysis of the amplified products disclosed four to eight bands ranging from 700 to 1100 in each sample.

1.3.2.2.3 PCR-RFLP

PCR-RFLP is a PCR- based method for typing of microorganisms. A target sequence is generally 1 to 2 kb long and known to show polymorphism among strains of species of interest, is amplified with primers specific to a particular virulence region. The amplified product is cut with a suitable restriction endonuclease which is choosen on the basis of the known DNA base composition of target sequence of interest. Digested DNA is then visualized directly onto an agarose gel with ethidium bromide staining. Although it is a rapid, simple and reproducible technique, PCR-RFLP typing has shown so far only moderate discriminination (Strulens *et al.*, 1998).

PCR-RFLP assay was used in the study of Otranto *et al*. 2003 to differantiate *Hypoderma bovis* and *Hypoderma lineatum*. Genomic DNA extracted from 10 specimens of *H. bovis* and *H. lineatum*, and PCR was performed using conserved cytochrome oxidase I (COI) primers (UEA7-UEA10). PCR products were purified and sequenced, then by using appropriate computer packages inter-specific and intra-specific differences was detected among the sequences. Given the intra-specific polymorphisms at particular sites in the regions investigated, restriction enzymes were selected that cleaved at sites with no polymorphism: acordingly, among the potential enzymes assessed against *H. bovis* and *H. lineatum* COI sequences, *Bfa* I ,*Hinf* Iand *Taq*

I enzymez were chosen. Samples were digested ovenight at 65°C for Taq I and at 37°C for *Bfa* I and *Hinf* I. Comparisons of H. bovis and H.lineatum sequences revealed an inter-specific variation rate of 8.5%, while intra-specific polymorphism were deected at six nucleotide positions in H. bovis and two nucleotide position in H. lineatum (intra-specific variation rate of 0.87 and 0.29 respectively). The results showed that the COI gene region examined was useful for the differantiotion of H. bovis and H. lineatum and that a PCR-RFLP assay is a practical tool for their identification, offering diagnostic and epidemiological instruments for the study of cattle grub infestation.

1.3.2.2.4 Repetitive PCR (Rep-PCR)

The term rep-PCR refers to the general methodology involving the use of oligonucleotide primers based on short repetitive sequence elements that are dispersed throughout the prokaryotic kingdom. These elements appear to be conserved among many members of the Enterobacteriaceae and other bacterial species (Versalovic, Koeuth and Lupsky *et al.*, 1991).

Consensus sequence probes matching two such repetitive elements, the 126 bp enterobacterial repetitive intergenic consensus (ERIC) sequence and 38 bp repetitive extragenic palindromic (REP) sequence, both hybridize to genomic DNA from Enterobacteriaceae, other related Gram-negative bacteria. Outwardly directed fingerprints following PCR amplification of genomic DNA from microorganisms which contain these sequences. Differences in band sizes following electrophoresis on agarose gel apparently represent polymorphisms in the distances between repetitive sequence elements in different genomes (Versalovic *et al.*, 1992)

1.3.2.2.5 Randomly Amplified Polymorphic DNA (RAPD)

The randomly amplified polymorphic DNA (RAPD) is PCR-based DNA fingerprinting technique using single, arbitrarly designed, short (8-10 bp) nucleotide sequence as primer. Therefore, no prior sequence information of target DNA is needed, and lower annealing temperature (30-40°C) are used in RAPD (Williams *et al*., 1990). At sufficiently low temperature, the arbitrary primer bind to many sequences with a varity of mismatches on the genomic DNA template (McClelland *et.al*., 1990). The amplification process is initiated on those genomic regions in which two priming events occur on opposite DNA strands within a distance not exceeding a few thousand nucleotides. The outcome of the amplification reaction is determined by competition in which those pruduct representing the most efficient pairs of priming sites separted by the most easily amplifiable sequences will prevail (McClelland *et al*., 1995). It should be emphasized that these results do not imply that all amplifications are the results of perfect pairing between the primer and the DNA template. The number of DNA segments amplified from bacterial samples with much smaller genomes can only be explained on the basis of mismatch between the primer and the DNA template (Williams *et al*., 1990). As shown in Figure 1.1., two primers must anneal to the template DNA on opposite strands, and in opposite orientations such that they face each other, in order to achieve synthesis of both strands of the DNA segment which lies between the two primers. Furthermore, primers must anneal within a reasonable distance of one another, in order for the succesful PCR.

All classes of mutations (substitutions, insertions, deletions, invertions) can be potentially detected by means of random amplification fingerprinting even though most of the detected polymorphisms are supposed to be caused by substitutions. Single base substitutions within the primer binding site may prevent amplification by introducing a mismatch; this supported by the observation that most single base changes in a primer sequence result in complete change in the amplification pattern (Williams *et al*., 1990).

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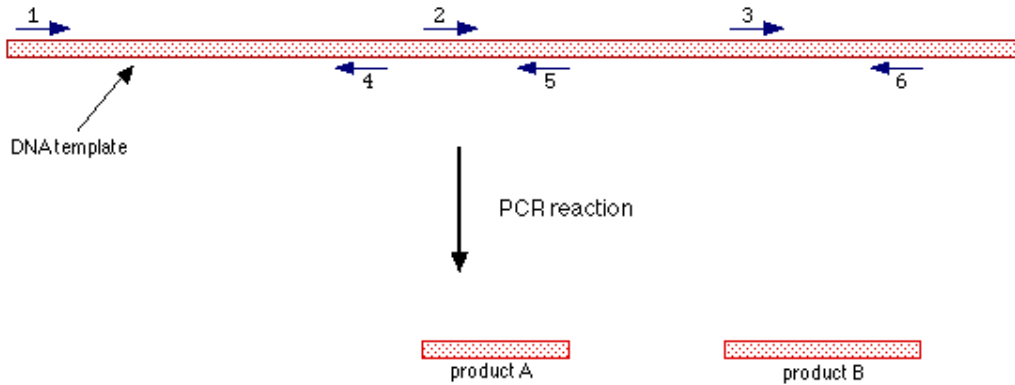
Figure 1.1. Schematic representation of RAPD-PCR

Reaction products are resolved by gel electrophoresis, and patterns generated from different genomic DNAs (e.g, DNAs from different individual or strains) using the same primer are easily compared to identify possible differences (i.e., DNA fragments that are amplified from genomic DNA but not from another). Any difference between the patterns of amplified fragments reveal a polymorphism in that it arises from a difference in template sequence which inhibits primer binding or otherwise interferes with amplification of the corresponding fragment.

The RAPD technique has further advantages over other systems of genetic documentations, because in RAPD, no previous genetic and molecular knowledge is required, a universal set of primers of arbitrary nucleotide sequence can be applied, a large number of primers are available, and only small amount of DNA is required. No southern blotting or radioactive probes are required, and the protocol is relatively quick and simple. RAPDs allow quick detection of usually 50-60 polymorphic DNA markers in 4-5 amplification reactions and speed in the assay of high number of samples. The ease and simplicity of RAPD technique make it ideal for genetic mapping, plant and animal breeding applications, DNA fingerprinting, with particular utility for studies of population genetics (Williams *et al*., 1990).

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RAPD fingerprinting was caried out by using four arbitrary primers, OPA-10, OPR-03, OPI-06 and OPJ-09, to assess the genetic diversity of twenty-six *Salmonella enterica* isolates which were obtained from hospital laboratories and commercial poultry producers in Mauritius. All clinical isolates were subjected to biochemical and serological testing for confirmation. Primer OPA-10 produced a single clear band of 1 kbp with all of the *Salmonella* isolates. Samples O and P did not produce the same bands. A larger number of bands were observed with primer OPR-03. A clear band of approximately 2.2 kbp was obtained with all isolates except O, P, Q and R. Similarly, each produced a band of 1 kbp. Primer OPJ-09 produced two clear bands of approxmately 2250 bp and 1800 bp from all the isolates of *Salmonella* except Q, X, Z, and A. Primer OPI-06 produced greatest number of bands, ranging between 220 kbp to 2200 kbp. RAPD analysis of *Salmonella* isolates from Mauritius showed that hey are genetically diverse. Four primers were selected for testing the samples by amplification. From all four banding patterns, it is clear that isolates O and P, which were mistakenly identified as *Salmonella* by biochemical and serological analyses, were later confirmed to be *Proteus* species and not *Salmonella*. In this respect, primer OPA-10 is potentially usefull for the identification of and differentiation of the *Salmonella* species from other Gram-negative bacteria (Khoodoo *et al*., 2002).

RAPD analysis was performed for the molecular genetic typing of 30 *Salmonella enterica* subsp. *enterica* strains isolated from chickens and duck in Thailand. Six different primers were tested for their discriminnatory ability. They found that most of the different serovars produced different RAPD types using only one primer. For example, all eight *Salmonella* Enteritidis showed identical patterns using primer 4 and this pattern was different from the pattern with primer 4 all other tested strains. Of these same eight strain five showed an identical pattern with primer 1, while remaning three strain showed individual patterns with primer 1. Using all six primers the RAPD could differentiate

between all of the *Salmonella* Enteritidis isolates (Chansiripornchai *et al.*, 2000).

Another study was prepared by Soto *et al.* (1999) for typing of *Salmonella enterica* strains assigned to 12 serotypes. The series of organisms used included 235 strains (326 isolates) collected mainly from clinical samples. RAPD reaction were carried out with the following three primers; primer S, primer B, primer C, respectively. With primer S, 21 amplified DNA band profiles were differentiated; each of these profiles included 2 to 5 fragments which were 250 to 2000 bp long. With primer B, 14 amplified DAN band profiles were differentiated; each of these profiles included 4 to 7 fragments which were 400 to 2500 bp long and with primer C, 40 amplified DNA band profiles were obtained. Most of these profiles were defined by considering only bands that were 1700 bp long or smaller. Different amplified fragments appeared to be characteristic of different serotypes.

In anather study, RAPD described to analyse 23 *Salmonella* spp. and 16 non-Salmonella spp. By using single oligonucleotide primer (du-primer) synthesized on the basis of N-terminal amino acid sequence of *Salmonella* dulcitol 1-phosphate dehydrogenase (D1PDH). Among three to five prominent bands produced the two bands at about 460 and 700 bp were detected all the strains of *Salmonella*, but some of the non-*Salmonella* bacteria showed no bands and the others showed different band patterns with some primer and same conditions (Miyamoto *et al.*, 1998).

Previous study was performed by Ören in 1997 to evaluate the efficiecy of RAPD-PCR method for identification of *Salmonella* serotypes. For this purpose 10 different random oligonucleotide primers, 8-10 bp in size, were used with 14 different *Salmonella* serotypes and 16 non-*Salmonella* strains. Three of these primers which were primer 7, primer 6, and primer 3 gave distingusihable band patterns. Primer 7 gave 550 bp strong amplification band with 11 of the 14 *Salmonella* serotype, but some of the non-*Salmonella* strains

gave same band. Primer 3 and primer 6 produce different fragments with *Salmonella* serotypes. Primer 3 gave a distinguishable amplification pattern and strong band with *Salmonella* Typhimurium. According to this result, it was decided that further analysis should be performed with different *Salmonella* Typhimurium isolates and also other *Salmonella* serotypes, especially closely related serotype *Salmonella* Enteritidis to decide whether this band can be used as a specific marker for detection and identification of *Salmonella* Typhimurium.

1.4 Aim of The Study

Aim of this study was to confirm the results of Ören (1997) and to detect the specific DNA marker for serotype *Salmonella* Typhimurium using Randomly Amplified Polymorphic DNA (RAPD) which is a PCR-based DNA fingerprinting approach, and to prove that other *Salmonella* serotypes especially *Salmonella* Enteritidis does not contain this specific DNA marker, since *Salmonella* Enteritidis shows very similar disease symptoms with *Salmonella* Typhimurium and it can easly be confused with *Salmonella* Typhimurium.

RAPD-PCR is commonly used for the differentiation of closely related strains of bacteria (Busse *et al.*,1996) since RAPD has the potential to detect polymorphism throughout the entire genome as compared other techniques (Hilton et al., 1998). Furthermore, RAPD is more suitable than other techniqes because it does not require prior knowledge of target DNA and it uses short (9-10 bases) primers with small amount of starting DNA. Because of these advantages RAPD is selected to differentiate *Salmonella* Typhimurium from other *Salmonella* serotypes in this study.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacterial strains

Bacterial strains used in this study are presented in Table 2.1 and Table 2.2.

2.1.2 Chemicals and Enzymes

The list of chemicals and enzymes used and their suppliers are given in Appendix A.

2.1.3 Growth Media, Buffers and Solutions

The preparation of the growth media, buffers and solutions used are given Appendix B.

2.2 Methods

2.2.1 Maintanance and Cultivation of Bacterial Strains

All *Salmonella* strains were grown in Tyriptic Soy Broth (TSB) (Appendix B) at 37°C for 1 day. Cultures were stored on Tyriptic Soy Agar

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(Appendix B) slants at 4°C. For longer periods of maintenance bacterial strains kept in 20 % glyserol and microbank at -80°C.

Salmonella strains used in this study and their suppliers were shown in Table 2.1 and Table 2.2.

Table 2.1. Salmonella Typhimurium strains was used in the study.

Strain Designation	Name of the Species	Area of Isolation	Supplier
A6	S.Typhimurium	Ankara	Numune Hospital, Dept. Bacteriol.
A20	S.Typhimurium	Ankara	Numune Hospital, Dept. Bacteriol.
H28	S. Typhimurium	Bursa	Uludağ Univ. Fac. Of Medicine
H29	S. Typhimurium	Bursa	Uludağ Univ. Fac. Of Medicine
H31	S. Typhimurium	Bursa	Uludağ Univ. Fac. Of Medicine
H33	S. Typhimurium	Bursa	Uludağ Univ. Fac. Of Medicine
H49	S. Typhimurium	Bursa	Uludağ Univ. Fac. Of Medicine
ST4	S. Typhimurium	Ankara	METU Dept. of Biology
*RSKK58	S. Typhimurium	Ankara	Refik Saydam Kültür Kolleksiyonu
2/15 *(RSKK28)	S. Typhimurium	France	Institute of Pasteur
2/16 *(RSKK29)	S.Typhimurium	France	Institute of Pasteur
8391 *(NCTC02010)	S. Typhimurium	England	National Culture Type Collection

* Salmonella Typhimurium isolates used in this study were shown in Table 2.1. Of these, four strains were obtained from Refik Saydam Culture Collection.

Table 2.2. *Salmonella* Enteretidis and other *Salmonella* serotypes strains used in the study.

Strain Designation	Name of the Species	Area of Isolation	Supplier
H3	*S*. Enteretidis	Ankara	Numune Hospital, Dept. Bacterio.
H12	*S*. Enteretidis	Ankara	Numune Hospital, Dept. Bacterio.
H16	*S*. Enteretidis	Ankara	Numune Hospital, Dept. Bacterio.
H19	*S*. Enteretidis	Ankara	Numune Hospital, Dept. Bacterio.
H20	*S*. Enteretidis	Ankara	Numune Hospital, Dept. Bacterio.
H21	*S*. Enteretidis	Ankara	Numune Hospital, Dept. Bacterio.
H55	*S*. Enteretidis	Bursa	Numune Hospital, Dept. Bacterio.
H59	*S*. Enteretidis	Bursa	Numune Hospital, Dept. Bacterio.
E8	*S*. Enteretidis	Bursa	Uludağ Univ., Dep. of Microbiol.
E9	*S*. Enteretidis	Bursa	Uludağ Univ., Dep. of Microbiol.
E10	*S*. Enteretidis	Bursa	Uludağ Univ., Dep. of Microbiol.
H38	*S*. Agona	Bursa	Uludağ Univ., Dep. of Microbiol.
H23	*S*. Baildon	Ankara	Ankara Univ., Dep. of Biology
H32	*S*. Havana	Bursa	Uludağ Univ., Dep. of Microbiol.
CH32	*S*. Havana	Bursa	Uludağ Univ., Dep. of Microbiol
T6	*S*. Havana	Ankara	METU, Dep. of Biology
T11	*S*. Havana	Ankara	METU, Dep. of Biology
T12	*S*. Havana	Ankara	METU, Dep. of Biology
T58	*S*. Havana	Ankara	METU, Dep. of Biology
E1	*S*. Kedougou	Ankara	Köytür
E2	*S*. Kedougou	Ankara	Köytür
A18	*S*. Ouakam	Ankara	Lalahan Animal Health Institute
A24	*S*. Ouakam	Ankara	Numune Hospital, Dept. Bacterio.

2.2.2 Small Scale Genomic DNA Isolation

Isolation of genomic DNA was performed by modifiying method of Jones and Barlet, 1990. According to this method bacterial cells from an overnight culture previously grown at 37°C in 10ml of TS broth (Appendix B) were transferred to 1.5ml eppendorf tube and spinned at 13000 rpm for 2 min, after supernetant was removed new 1.5ml cell culture was added onto the pellet and again spinned at 13000 rpm for 2 min. Then pellet was resuspended in 467μl TE buffer (Appendix B) by repeated pipeting, 30μl of 10% SDS (Appendix B) and 3μl of 20 mg/ml proteinase K were added and this mixture was incubated at 37°C for 1 hour. Following 1hour incubation equal volume (500μl) phenol/chloroform/isoamylalcohol was added and mixed well by inverting the tube untill the phases were completely mixed, and then tube was spinned 13000 rpm for 2 min. The upper aqueous phase phase containing nucleic acids was carefully transferred to a new tube and phenol/chloroform/isoamylalcohol extraction was repeated untill the interphase was clear. The aqueous phase obtained after the last in the series of deproteinization was mixed with 0.1X volume 3M sodium acetate and ethanol precipitation was performed by adding 2X volume ice-cold 95% ethanol. DNA was precipitated overnight at -20°C and collected by centrifugation at 15000 rpm for 15 min. Finally, the supernatant was discarded , the pellet was washed with 1ml of ice-cold 70% ethanol to eliminate salt and centrifuged another 30 second at 15000 rpm. Tube was allowed to air-dry till al the ethanol was evaporated. DNA was dissolved in 30-50μl TE buffer and stored at -20°C.

The resulting DNA concentration was determined by comparing the intensity of the band with that of the bands of known concentration on agarose gel.

2.2.3 RAPD-PCR Amplifications

A 50 μl PCR mixture contained;

- 10X reaction buffer to give a final cocentration of 1X.
- 0.25 mM MgCl
- 2 mM dNTP mix 2 units of Taq Polymerase
- 200 pmoles of primer
- 0.05 μg DNA
- Sterile double distilled water to give afinal volume of 50μl

30 μl of mineral oil is added to prevent evaporation of PCR mixture.

Amplifications were performed with Primer 3 (RAPD 9.1) on a thermal cycler (TECHNE Progene) according to the following cycle in Table 2.3.

Table 2.3. RAPD-PCR Conditions for Primer 3 (RAPD 9.1)

90°C	5 min	
89°C	1 min	
32°C	1 min	35 cycle
72°C	1.5 min	
50°C	3 min	

Reamplification of 700 bp band were performed with Primer CA 700 to add restriction side to the band for ligation according to the following cycle in Table 2.4.

Table 2.4. RAPD-PCR Conditions for Primer CA 700

90°C	5 min	
89°C 32°C 72°C	1 min 1 min 1,5 min	10 cycle
89°C 55°C 72°C	1 min 1 min 1,5 min	25 cycle
50°C	3 min	

2.2.4 Agarose Gel Electrophoresis and Visualization of DNA and PCR Products

0.8% agarose gel (Appendix B) was used to analyse genomic DNA and PCR amplification products. The gel was melted and cooled to 50-55°C. After addition of ethidium bromide, which is a fluroscent dye, intercalates between the bases of nucleic acids and fluoresces when it is exposed to UV light, at concentration of 0.5 µg/ml. The gel was poured into mould and solidify for about 15 min. Then it was placed in the electrophoresis tank filled with 1X TAE buffer (Appendix B). Electrophoresis was performed at 70 V for 60 min. Then the gel was visualized on UV transilluminator at 320 nm. Gel were photographed by Nicon Coolpix 4500 digital photograph machine.

2.2.5 Extraction of PCR Product from Agorose Gel

The desired PCR fragment was recovered using a DNA Extraction Kit (Fermentas) by cutting the DNA fragment from an agarose gel after agarose gel electrophoresis (section 2.2.4). The volume of excised gel slice which was

containing desired band was determined by weight. 3 volumes of binding solution was added to 1 volume of sample and incubated at 55ºC for 5 minutes to dissolve agarose. Then the resuspended silica powder suspension was added as 2 µl per 1µg of DNA. The mixture was incubated at 55°C for 5 minutes and was mixed in every 2 min by vortexing to keep silica powder in mixture. After centrifugation of mixture at 13 000 rpm for 5 seconds the supernatant was discarded and 500 µl ice cold extraction wash buffer (Appendix B) was added to the pellet and the pellet was resuspended by vortex. The tube was centrifuged at 13 000 rpm for 5 seconds and supernatant was discarded. This washing procedure was repeated three times. During each washing the pellet was resuspended completely.

After last washing the supernatant was removed and the pellet was air-dried for 15-20 min. The pellet was resuspended in double distilled water and incubated at 55°C for 5 min. The tube was centrifuged at 13 000 rpm for 30 second and then the supernatant which was containing DNA removed to the new tube. Finally, extracted DNA was run on agorose gel with a marker of known concentration to determine the concentration by compairing the intensity of band with the marker.

2.2.6 Restriction Digestion of PCR Product

After purification of PCR products, DNA concentration was calculated according to reaction mixture volume and depending on this concentration the amount of enzyme was calculated.

For 20 µl of reaction mixture the following reagents was added into the microcentrifuge tube:

- 2 µl of 10X reaction buffer was added to final concentration of 1X
- 0.1 to 5 µg DNA
- Appropriate amount of enzyme

- Enough double distiled water to obtain final volume to 20 µl

Finally reaction mixture was mixed, and 15 µl mineral oil was added to prevent evaporation. The reaction was incubated overnight at 37°C which is specific for the enzyme *Hind* III

2.2.7 Ligation

After the 5 µg plasmid DNA was digested with appropriate restriction enzyme as explained in section 2.2.6 the plasmid was dephosphorylated with alkaline phosphatase to prevent self ligation of vector by removing 5' phosphate.

50µl reaction mixture for the dephosphorylation of plasmid DNA contains reagents as in below:

- Appropriate 10X restriction enzyme buffer to give a final concentration of 1X
- Solution of DNA (1 to 20 pmol DNA termini = 0.88 to 17.5 µg of linear pUC19)
- 0.5 to 2 units calf intestine alkaline phosphatase
- Enough double distilled water to adjust final volume to 50µl

The reaction was incubated at 37°C for minimum 2 hours to overnight. After incubation period equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added into the tube. The mixture was centrifuged at 13 000 rpm for 10 minutes, upper phase was transferred to a new tube. DNA was precipitated by adding 0.1 volume sodium acetate (3 M, pH = 5.2) (Appendix B), and 2.5 volume cold absolute ethanol. The mixture was stored overnight at -20°C. Then the tubes were centrifuged at 13 000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed with 100 µl 70 %

ethanol (Appendix B). The tubes were centrifuged at 13 000 rpm for 3 minutes and the supernatant was discarded. The pellet was air-dried for 20 minutes.

Afterwards the PCR fragment to be ligated was also digested with the same restriction endonuclease according to the procedure explained in section 2.2.6., phenol:chloroform:isoamylalcohol extraction was also applied to the fragment and upper phase was transferred to a new tube. The mixture was stored overnight at –20°C by adding 0.1 volume sodium acetate (3 M, pH = 5.2) (Appendix B), and 2.5 volume cold absolute ethanol for precipitation. Then the tube was centrifuged at 13 000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed with 100 µl 70 % ethanol (Appendix B). The tube was centrifuged at 13 000 rpm for 3 minutes and the supernatant was discarded. The pellet was air-dried for 20 minutes.

Ligation mixture was prepared with below reagents in microfuge tube:

- Sterile double distilled water to give a final volume of 20 µl
- 10X ligation buffer to give a final concentration of 1X
- 1 mM ATP
- 1 unit T4 DNA ligase
- 0.1 µg plasmid
- µg insert is calculated according to the following equation
  molar ratio of insert to plasmid was selected as 3:1

$$\frac{\text{fragment size (bp)}}{\text{vector size (bp)}} \quad \text{x} \quad \frac{3}{1} \quad \text{x} \quad 0.1 \text{ µg plasmid}$$

The ligation mixture was incubated at 16°C overnight. 5 µl ligation mixture was transformed to a suitable strain of competent *E. coli* according to the transformation procedure explained in section 2.2.8.2. The transformed cultures were plated on 2YT ampicilin plates which were containing 100 µg/ml

ampicilin spreaded with 100 µl 100 mM IPTG (Appendix B) and 50 µl 2 % X-gal prior to plating and plates were incubated at 37°C for 30 minutes for the media to soak in IPTG and X-gal. Then transformed culture plates was incubated overnight at 37°C. The white colonies which contains insert were picked and the presence of insert confirmed by the plasmid isolation procedure explained in section 2.2.9.

2.2.8 Transformation of *E.coli XL1 Blue MRF*

2.2.8.1 Preparation of Competent Cells

E. coli XL1 Blue MRF cells were cultivated in 5 ml 2YT medium supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ (Appendix B), overnight at 37ºC with shaking. Then, 100 ml of 2YT medium supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ (Appendix B) was inoculated with 1 ml of an overnight culture of *E. coli XL1 Blue MRF* cells and incubated at 37°C until the OD_{550} reached 0.4-0.5. The culture was dispensed into 2 falcon tubes. The tubes were chilled on ice for 10 min and centrifuged at 6000 rpm for 5 min at 4°C. Then, the supernatant was discarded and the cells were resuspended in a total volume of 50 ml (25 ml for each tube) ice-cold Solution A (Appendix B), kept on ice for 15 min, centrifuged at 6000 rpm for 5 min at 4°C. Again, the supernatant was discarded, the cells were resuspended in a total volume of 7 ml (3.5 ml for each tube) ice-cold Solution A (Appendix B) and 100 % sterile glycerol was added to a final concentration of 20 % (700 µl for each tube). Finally, 300 µl aliquots were dispensed into eppendorf tubes and stored at -80°C.

2.2.8.2 Transformation of Competent Cells

5 µl plasmid was mixed with 50 µl TE buffer (Appendix B). This mixture was mixed with one batch of competent cells (300 µl) and kept on ice for 30 min. The mixture was transferred into a 42°C water bath for precisely 2

min. Then, 1 ml 2YT medium supplemented with 0.2% (w/v) maltose and 10 mM MgSO$_4$ (Appendix B) was added and kept at 37°C for 1 hr. 50-200 µl aliquots were finally spread on 2YT ampicilin agar plates (Appendix B) that were incubated with 100 µl 100 mM IPTG (Appendix B) and 50 µl 2 % X-gal (Appendix B) before spreading. Spreaded plates incubated overnight at 37°C.

2.2.9 Plasmid Isolation

Alkaline lysis extraction procedure was used for the plasmid isolation. 5 ml of 2YT broth (Appendix B) with ampicilin at a concentration of 100µg/ml was inoculated with a single colony of transformed bacteria in a falcon tube and incubated at 37°C overnight with vigorus shaking. The cells were centrifuged at 6000 rpm for 8 min and the supernatant was discarded. The bacterial pellet was resuspended in 200 µl Solution I (Apppendix B) and transfered to the clean eppendorf tube and incubated at room temperature for 15 min. Then 200 µl Solution II (Apppendix B) was added, the contents was gently mixed by inverting the tube 6-7 times and tube was stored on ice preciesly for 5 min. After adding 200 µl Solution III (Apppendix B), the tube 6-7 times and tube was stored on ice for 15 min and was centrifuged at 13 000 rpm for 10 min at 4°C. When centrifugation was completed supernatant was transferred to a new eppendorf tube and by adding 2 volume of ice-cold 96% ethanol was stored for 10 min at -20°C. Again tube was centrifuged at 13 000 rpm for 10 min, pellet was resuspended in 200 µl NE buffer (Apppendix B) and incubated for 1 hour on ice. At the end of 1 hour tube was centrifuged at 13 000 rpm for 15 min at 4°C, supernatant transferred to a new eppendorf tube and by adding 2 volume of ice-cold 96% ethanol (Apppendix B) stored at -20°C for 10 min. Finally, tube was centrifuged at 13 000 rpm for 10 min and the plasmid DNA was washed with 1 ml of 70 % ethanol (Appendix B), centrifuged at 13 000 rpm for 5 min. The supernatant was removed, the pellet was air dried for 5 min, and redissolved in a 15 µl of double distilled water. To the dissolved plasmid DNA solution, 10 µl, 10 mg/ml DNase free RNase (Appendix B) was added and incubated at 37°C for 2 hours.

CHAPTER 3

RESULTS AND DISCUSSIONS

RAPD-PCR was used during this study for different *Salmonella* serotypes and the strategy of RAPD-PCR application was given Figure 3.1.

First, the *Salmonella* serotypes were cultivated separately according to the section 2.2.1. Then DNA isolation was performed as mentioned in section 2.2.2. DNA concentration used in RAPD-PCR was optimized and 50 ng/reaction was found as an optimum for RAPD-PCR. Isolated DNAs of different *Salmonella* serotypes was used in RAPD-PCR with Primer 3 (RAPD 9.1).

The amplification results obtained from RAPD-PCR were analyzed by agarose gel electrophoresis and photographed. 700 bp long amplification fragment was found as a specific for serotype *Salmonella* Typhimurium. Then the cloning step for sequencing was performed and the strategy of cloning was determined.

3.1. Strategy of RAPD-PCR

The strategy of RAPD-PCR application was given Figure 3.1.

```
┌─────────────────────────────┐
│   Cultivation of organisms  │
└─────────────────────────────┘
               ⇩
┌─────────────────────────────┐
│  DNA isolation from bacteria│
└─────────────────────────────┘
               ⇩
┌─────────────────────────────┐
│  Optimization of genomic    │
│  DNA concentrations         │
└─────────────────────────────┘
               ⇩
┌─────────────────────────────┐
│  RAPD-PCR    with  arbitrary│
│  designed    Primer 3 (RAPD │
│  9 1)                       │
└─────────────────────────────┘
               ⇩
┌─────────────────────────────┐
│  Visualisation of bands on  │
│  agarose gel                │
└─────────────────────────────┘
               ⇩
┌─────────────────────────────┐
│  Bands are photographed     │
└─────────────────────────────┘
```
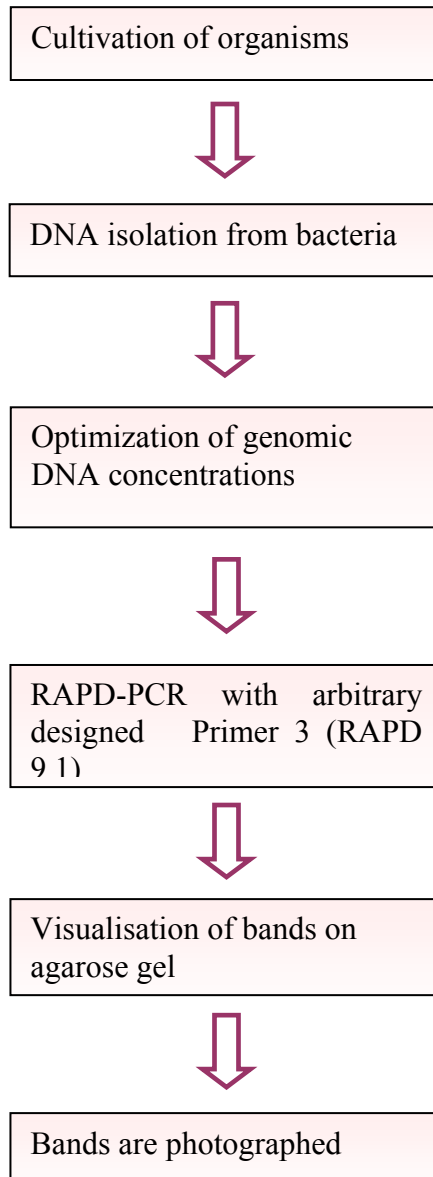
Figure 3.1. Strategy of RAPD-PCR

3.2 DNA Isolation Results

Genomic DNA isolation of all *Salmonella* serotypes was performed according to the method in section 2.2.2. Isolated genomic DNAs of different *Salmonella* Typhimurium strains shown in Figure 3.2. The concentrations of

isolated genomic DNA of different *Salmonella* Typhimurium strains were analyzed by agarose gel electrophoresis as explained section 2.2.2.
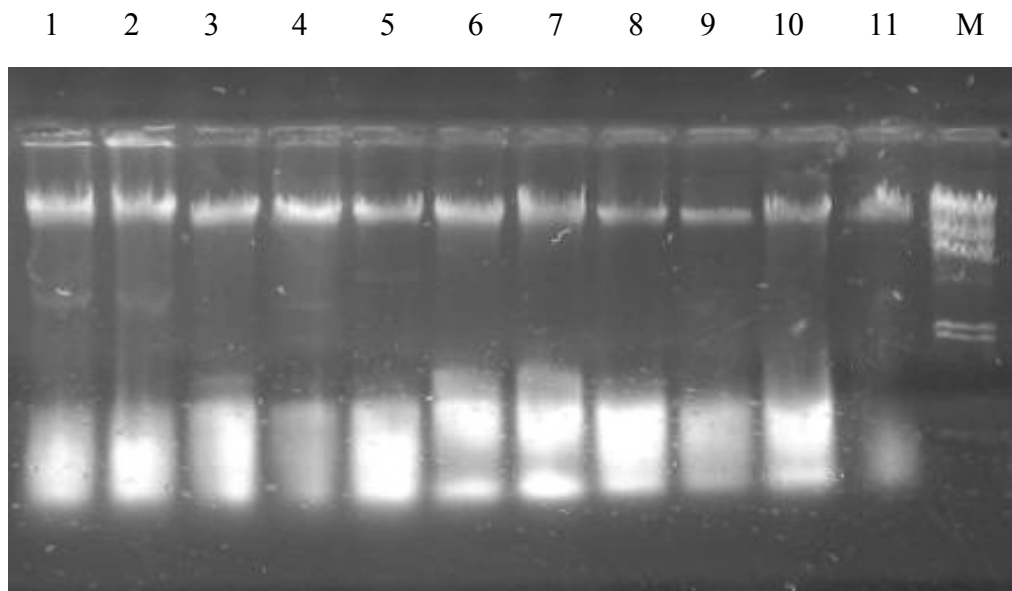
Figure 3.2. DNA isolation results of by agarose gel electrophoresis. **1,** *S.* Typhimurium A6; **2,** *S* .Typhimurium A20; **3,** *S.* Typhimurium H28; **4,** *S.* Typhimurium H29; **5,** *S.* Typhimurium H31; **6,** *S.* Typhimurium H33; **7,** *S.* Typhimurium H 49; **8,** *S.* Typhimurium R SKK 28; **9,** *S.* Typhimurium RSKK 29; **10,** *S.* Typhimurium RSKK 58; **11,** *S.* Typhimurium RSKK 02010 ; **M,** λDNA /*Hind*III marker.

Genomic DNA isolation results of different *Salmonella* Enteritidis strains shown in Figure 3.3.
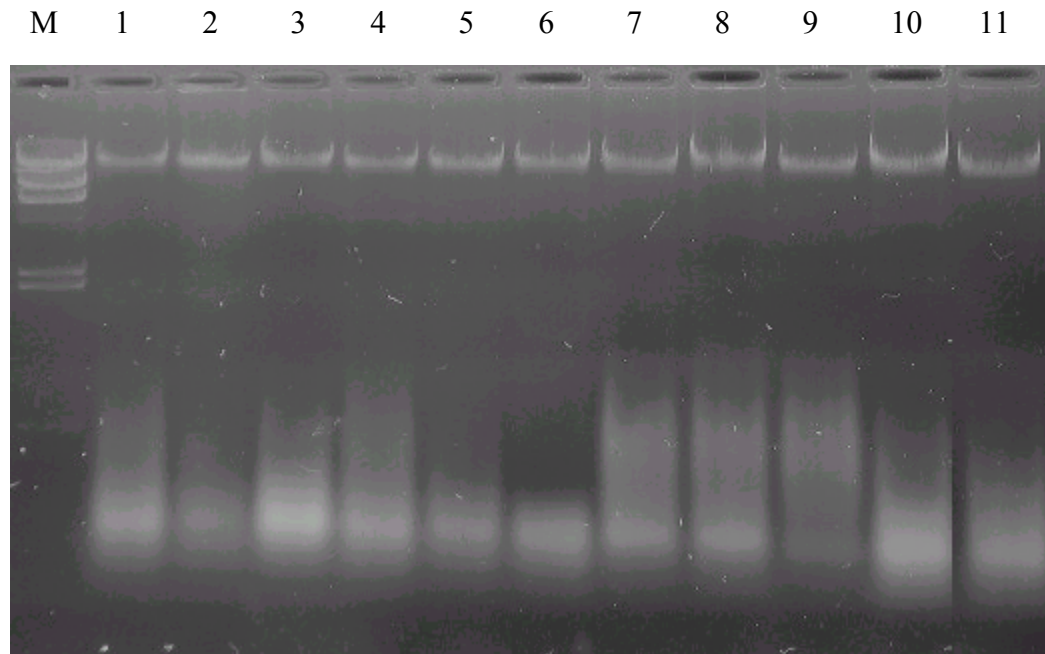
Figure 3.3. DNA isolation results by agarose gel electrophoresis, **M.** λDNA/*Hind*III marker; **1.** *S.* Enteritidis H3; **2.** *S.* Enteritidis H12; **3.** *S.* Enteritidis H16; **4.** *S.* Enteritidis H19; **5.** *S.* Enteritidis H20; **6.** *S.* Enteritidis H21; **7.** *S.* Enteritidis H55; **8.** *S.* Enteritidis H59 ; **9.** *S.* Enteritidis E8; **10.** *S.* Enteritidis E9; **11.** *S.* Enteritidis E10.

Genomic DNA isolation results of different *Salmonella* serotypes shown in Figure 3.4.
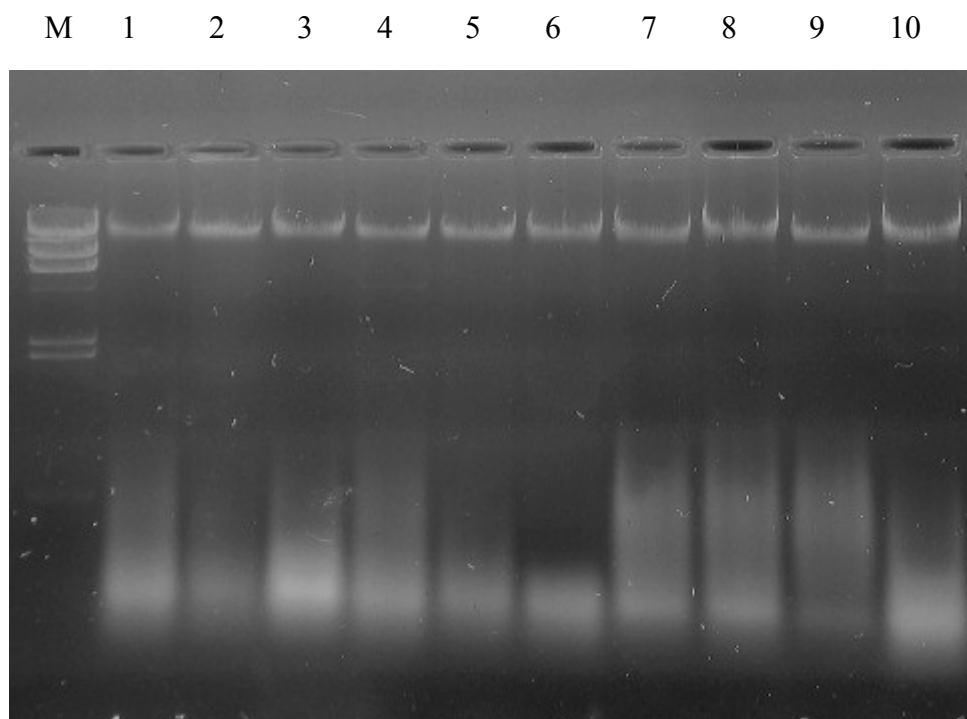
Figure 3.4. DNA isolation results by agarose gel electrophoresis, **M.** λDNA/ *Hind*III marker; **1.** *S.* Havana H32; **2.** *S.* Havana T6; **3.** *S.* Havana T11; **4.** *S.* Havana T12; **5.** *S.* Kedougou E1; **6.** *S.* Kedougou E2; **7.** *S.* Oukam A18; **8.** *S.* Oukam A24; **9.** *S.* Boildon H23; **10.** *S.* Agona H38.

3.3 Optimization of Genomic DNA Concentration Used in RAPD-PCR

Genomic DNA was isolated on a small scale by making slight modifications of the method of Jones and Barlet 1990, by this method DNA was succesfully isolated from all *Salmonella* serotypes. *Salmonella* Typhimurium DNA was used in RAPD-PCR with Primer 3 (RAPD 9.1) according to the given PCR conditions in order to optimize the DNA

concentration used in RAPD-PCR. Two different concentration were tried and optimum concentration was found as 50 ng/reaction (Figure 3.5).
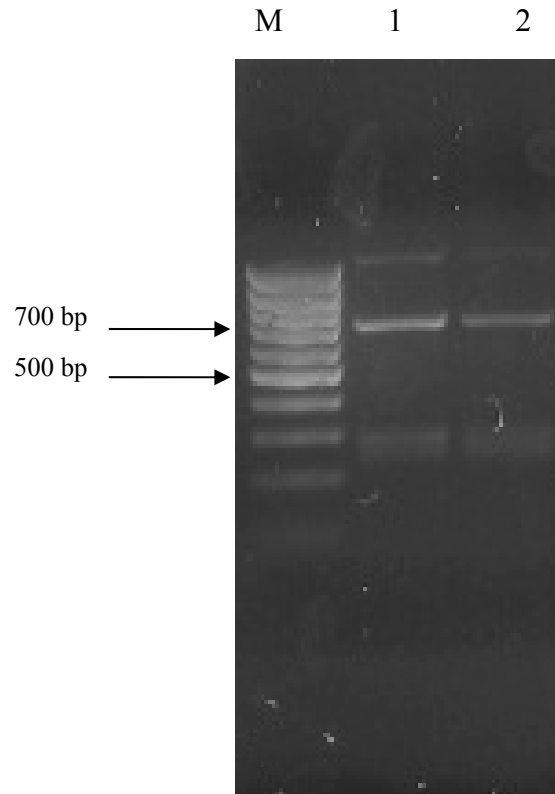
Figure 3.5. Optimization results; **M.** 100 bp DNA Ladder **1.** 50ng/reaction *Salmonella* Typhimurium DNA; **2.** 25ng/reaction *Salmonella* Typhimurium

The selection of appropriate primer and optimization of PCR conditions are of great importance for maximizing the discriminatory power and reproducibilty of RAPD analysis. Moreover, the standardization of DNA template concentration is of critical importance for avoiding artifact band patterns (Ellsworth *et.al.*, 1993).

3.4 Amplification Results by Using Short Oligonucleotide Primer

3.4.1 Primers Used in RAPD-PCR

In previous study performed by Ören (1997), 10 different randomly designed oligonucleotide primers had been screened for the DNA of 14 different *Salmonella* serotypes one of which was a *Salmonella* Typhimurium. Among these primers Primer 3 (RAPD 9.1) had produced a distingusihable band which was unique for serotype *Salmonella* Typhimurium. According to the results obtained by Ören, it was decided to perform detailed investigation with Primer 3 (RAPD 9.1) for serotype *Salmonella* Typhimurium.

Table 3.1. The sequence of primer 3 (RAPD 9.1)

Primer 3 (RAPD 9.1)	**5'-CGT GCA CGC-3'**

Because single primer was used in RAPD-PCR, automatic sequencing method was not proper for the sequencing of specific amplification product that was 700 bp long. As a result, it was decided to ligate 700 bp amplification product into a cloning vector and then to sequence ligated vector by using vector specific primers. Another primer CA 700 which was 20 bp in length was designed for ligation purpose. The primer CA 700 had *Hind*III cut side at 5'end to obtain cohesive ends for ligation onto the pUC19 vector having *Hind*III cut side . Reamplification of 700 bp band was performed with primer CA 700 by changing the PCR conditions as determined in section 2.2.3.

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Table 3.2 The sequence of primer CA 700

CA 700	5'- CGCAGAAGCTTCGTGCACGC -3'

RAPD primers are generally short oligonucleotide sequences 9-10 bp in length, between 50-80% G+C in composition, and contained no palindromic sequences (Beckmann *et.al.*, 1988). The GC content of Primer 3 (RAPD 9.1) was 77% and the GC content of CA 700 wasa 65%. and both of them were not contian polindromic sequences.

3.4.2 Amplification Results

Ören (1997) had screened 10 different random primers, 8-10 bp in size, against 14 different *Salmonella* serotypes, and 16 non-*Salmonella* strains. Only three of these primers, primer 7, primer 6, primer 3, had gave distinguishable band patterns. 550 bp strong amplification band had been obtained against 11 *Salmonella* serotypes with primer 7, but some of the non-*Salmonella* strains had also gave same band. Therefore, primer 7 was eliminated. Primer 3 and 6 had produced several distinct bands of sizes with *S.* Typhimurium DNA. Both primers had yielded different amplification patterns with other *Salmonella* serotypes. Only primer 3 had produced unique amplification band, 700 bp, and occasionally band pattern, from 800 bp to 1000 bp for only some isolates of serotype *Salmonella* Typhimurium.

According to these results, it was decided to carry out a comprehensive investigation with Primer 3 (RAPD 9.1) including higher numbers of *Salmonella* Typhimurium isolates. Of 12, 4 *S.* Typhimurium isolates would belong to internationally known culture collections. Amplification results of serotype S. Typhimurium were shown in Figure 3.7 and Figure 3.8.

Amplification results of 8 different *Salmonella* Typhimurium isolates with Primer 3 (RAPD 9.1) was shown in Figure 3.6.
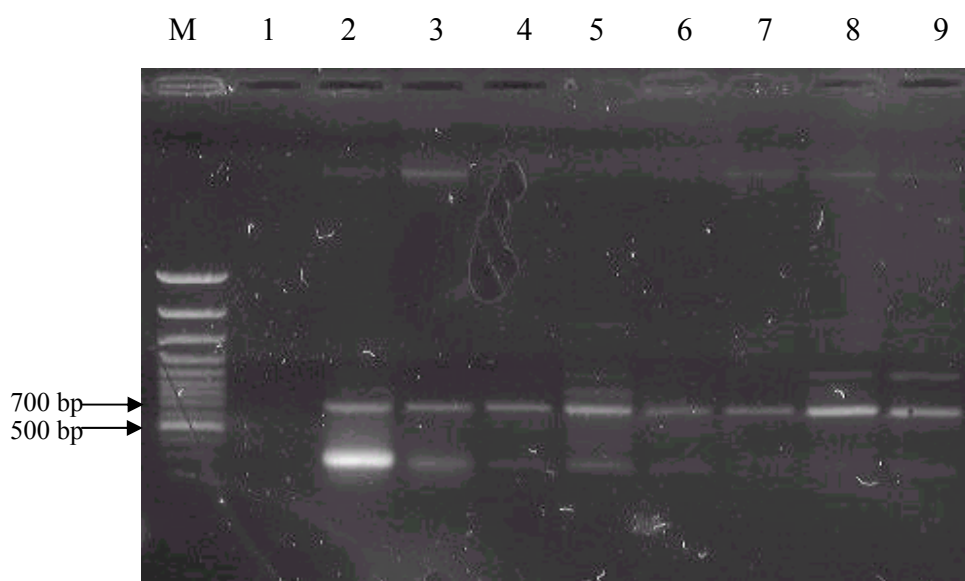
Figure 3.6. Amplification results of *Salmonella* Typhimurium; **M.** 100 bp DNA ladder plus marker; **1.** negative control; **2.** *S. typhimurium* A6; **3.** *S. typhimurium* A20; **4.** *S. typhimurium* H28; **5.** *S. typhimurium* H29; **6.** *S. typhimurium* H31; **7.** *S. typhimurium* H49; **8.** *S. typhimurium* ST24 .

All *Salmonella* Typhimurium isolates was used in this study gave distinguisihable amplification band at about 700 bp, also some strains of serotype *S.* Typhimurium gave additional weak bands at about 800 bp, 900 bp, 1000 bp and strong band at about 300 bp (Figure 3.6.).

The four *Salmonella* Typhimurium strains which belong to Institute of Pasteur and National Culture Type Collection (NCTC) were obtained from Refik Saydam Cultur Collection used to validate results obtained for other 8 *S.* Typhimurium isolates and they gave same strong amplification bands both at

about 700 bp and 300 bp and also other additional weak bands were observed for some of them (Figure 3.7.).
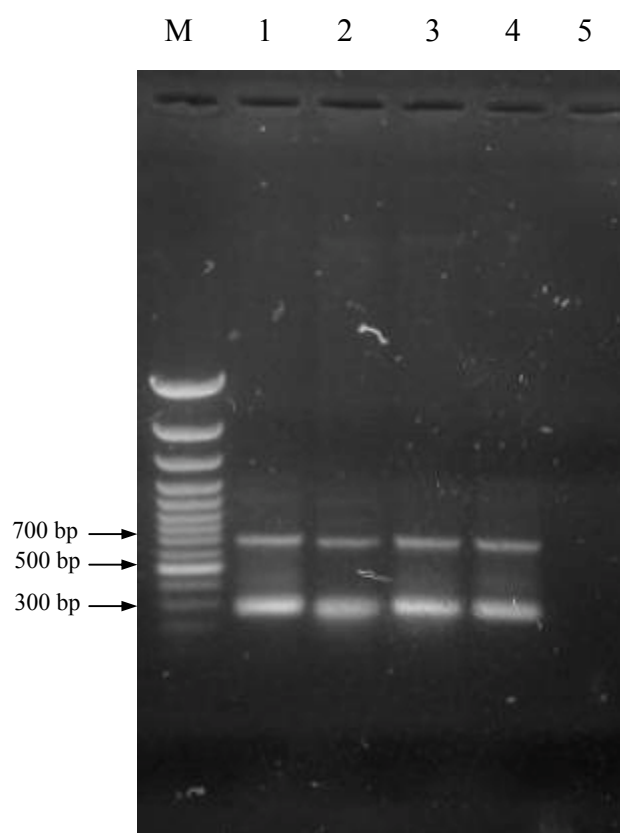
Figure 3.7. Amplification results of *Salmonella* Typhimurium; **M.** 100 bp DNA ladder plus marker; **1.** *S.* Typhimurium Institute of Pasteur 2/15 (RSKK 28); **2**. *S.* Ttyphimurium Institute of Pasteur 2/16 (RSKK 29); **3.** *S.* Typhimurium NCTC 8391 (RSKK 02010); **4.** *S.* Typhimurium RSKK 58; **5.** negative control.

Serotype *Salmonella* Enteritidis which is a very closely related serotype with *Salmonella* Typhimurium. They are pathogenic for both human and animals. In this respect, identification of serotype specific DNA marker for *Salmonella* Typhimurium and differentiation from Salmonella Enteritidis are very important both human health and food industry. Therefore, in order to

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check and express the specificity of amplification results of *Salmonella* Typhimurium, Primer 3 (RAPD 9.1) was applied to DNAs of different *Salmonella* Enteritidis isolates in RAPD-PCR (Figure 3.8.).

Amplification results of serotype *Salmonella* Enteretidis was shown in Figure 3.8.
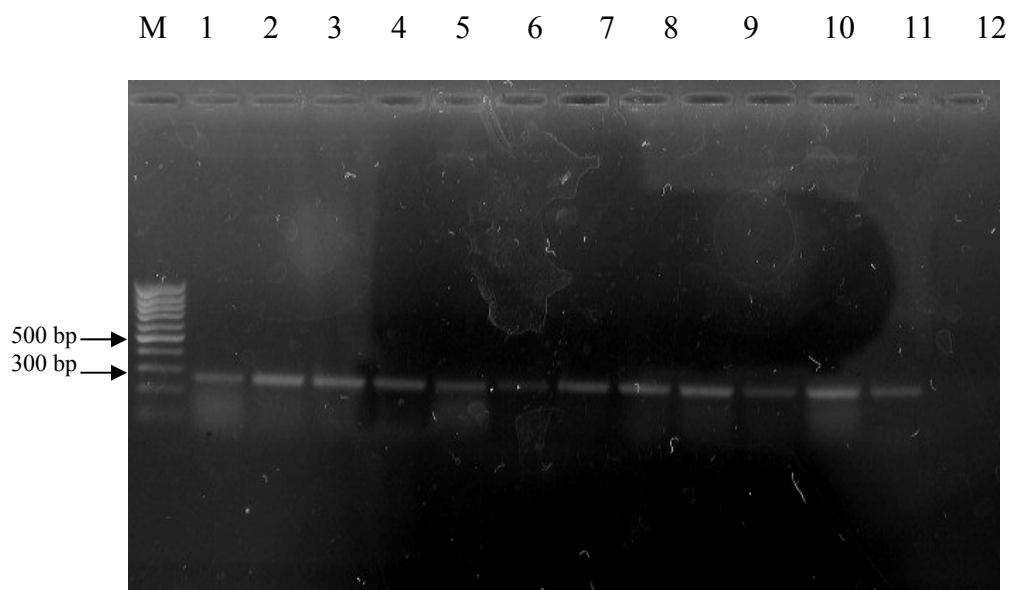
Figure 3.8. Amplification results of serotype *Salmonella* Enteretidis; **M.** 100 bp DNA ladder; **1.** *S.* Enteritidis H3; **2.** *S.* Enteritidis H12; **3.** *S.* Enteritidis H16; **4.** *S.* Enteritidis H19; **5.** *S.* Enteritidis H20; **6.** *S.* Enteritidis H21; **7.** *S.* Enteritidis H55; **8.** *S.* Enteritidis H59; **9.** S. Enteritidis E8; **10.** *S.* Enteritidis E9; **11.** *S.* Enteritidis E10; **12.** negative control

Amplification results of other *Salmonella* serotypes were shown in Figure 3.9.
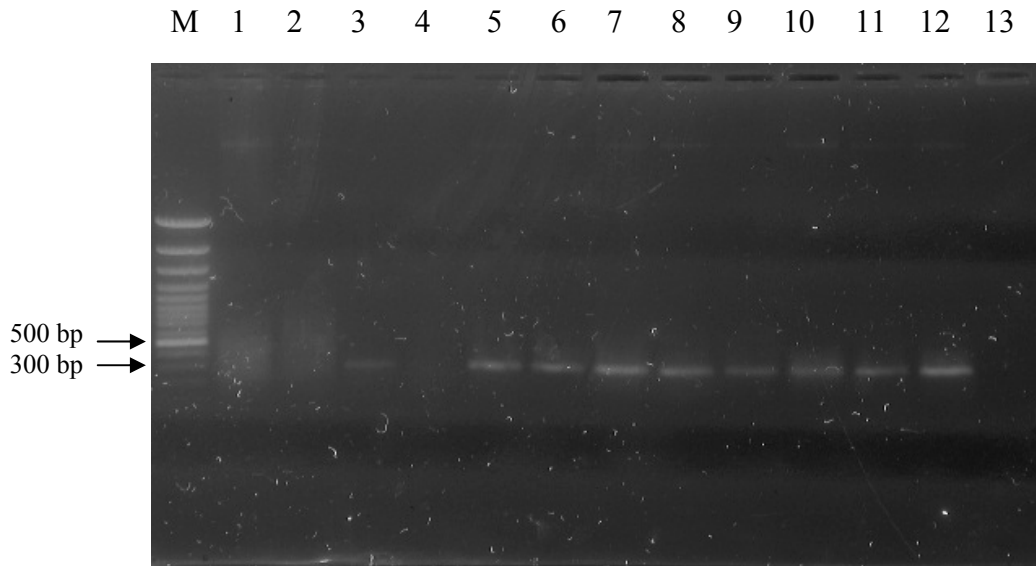
Figure 3.9. Amplification results of other *Salmonella* serotypes; **M.** 100 bp DNA ladder plus marker; **1.** *S.* Oukam A18; **2.** *S.* Oukam A24; **3.** *S.* Kedougou E1; **4.** *S.* Kedougou E2; **5.** *S.* Agona H38; **6.** *S.* Boildon H23; **7.** *S.* Havana H32; **8.** *S.* Havana CH32; **9.** *S.* Havana T6; **10.** *S.* Havana T11; **11.** *S.* Havana T12; **12.** *S.* Havana T58; **13.** negative control.

All serotype *Salmonella* Enteritidis isolates and some of the other *Salmonella* serotypes given in Table 2.2., produce 300 bp amplification band (Figure 3.8. and 3.9.) with Primer 3 (RAPD 9.1) under the same RAPD-PCR conditions with *Salmonella* Typhimurium in our study. Moreover, serotypes *S.* Typhi, *S.* Paratyphi B, *S.* Anatum, *S.* Strasbourg, S. Orion, *S.* Rissen had not produced 300 bp band with primer 3 (RAPD 9.1) in Ören's study. Therefore, it was found that 300 bp band could not be specific both *Salmonella* Typhimurium and for Salmonellae genus, so it was decided that 700 bp

amplification band could be used as a specific DNA marker for *Salmonella* Typhimurium.

The complete genome sequence study of *Salmonella* Typhimurium LT2, which is the principal strain for cellular and molecular biology in *Salmonella*, was performed by McClelland in 2001. The distribution of close homologous genes of *S.* Typhimurium LT2 in eigth related bacteria which were *Salmonella* Typhi, *Salmonella* Paratyphi A, *Salmonella* Paratyphi B, *Salmonella* Arizonae, *Salmonella* Bongori, *E. coli* K12, *E. coli* O157:H7 and *K. Pneumoniae*.

Genome sequence study of *Salmonella* Typhi, *E. coli* K12, *E. coli* O157:H7 was completed. *Salmonella* Typhi showed 89% homology with *Salmonella* Typhimurium LT2, *E. coli* K12 showed 71% homology with *Salmonella* Typhimurium LT2 and *E. coli* O157:H7 showed 73% homology with *Salmonella* Typhimurium LT2. 97% of genome sequences of *Salmonella* Paratyphi A and *K. Pneumoniae* was completed and they showed 89% and 73% homology with *Salmonella* Typhimurium LT2. In regard to other three enterobacteria, which were *Salmonella* Paratyphi B, *Salmonella* Arizonae, *Salmonella* Bongori, did not have genome sequence study. However, the microarray study result was available. According to the microarray results *Salmonella* Paratyphi B showed 92% homology with *Salmonella* Typhimurium LT2, *Salmonella* Arizonae showed 83% homology with *Salmonella* Typhimurium LT2 and *Salmonella* Bongori showed 85% homology with *Salmonella* Typhimurium LT2 (McClelland *et. al*., 2001).

Ören (1997) had used *S.* Typhi, *S.* Paratyphi B, *E. coli* and *K. Pneumonia* in RAPD-PCR with Primer 3 (RAPD 9.1), but none of them had given neither the 300 bp band nor the 700 bp band both of which were given by *S.* Typhimurium in our study. Specificity of the 700 bp band for *Salmonella* Typimurium in our study was consistent with the homology compairing study results of McClelland *et.al.*, 2001 since he had discovered that there were

nonhomolog regions on DNA among these bacteria. It was thougth that specific and polymorphic 700 bp region of *Salmonella* Typhimurium could be located at region that did not show homology with closely related *Salmonella* serotypes and enterobacteriaceae members.

After specific and polymorphic 700 bp band was found for *Salmonella* Typhimurium, this band was decided to be sequenced. Although the complete genome sequence study of *Salmonella* Typhimurium LT2 was reported, we could not obtain any result by the blast search since the length of Primer 3 (RAPD9.1) was too short for blast search. (www.ncbi.nlm.nih.gov/genomes/geblast.cgi?gi=1587&tax=99287)

3.5 Cloning of 700 bp Long Fragment for Sequenicing Purpose

As it is known, a single primer is used in RAPD-PCR , but this is not appropriate for the sequencing of resulted fragment by automatic sequencing approach. Therefore, 700 bp amplification band obtained by RAPD-PCR in this study could not be sequenced by automatic sequencing. Meanwhile, similar sequencing studies for RAPD results was surveyed. For example, a study was performed for the sequencing of RAPD-PCR fragments of *Gossypium barbadense* by Ghany *et.al*., (2003). Firstly, RAPD fragments were excised from the agarose gel and purified using Qiagen Gel Extraction kit (Qiagen, Germany). Then Purified RAPD DNA fragments were cloned in pCR 4-TOPO vector with TOPO TA cloning kit (Invitrogen, USA) in the competent *E. coli* strain TOPO 10 and plasmid DNA was isolated using QIA Spin mini-prep kit (Qiagen, Germany). Finally, plasmid DNA was sequenced in both directions using BigDye Sequencing Kit and ABI 3700 DNA sequencer (ABI, USA).

As a results, the shed light on these studies it was decided that the band was going to be cloned into the pUC 19 (Appendix D) to sequence. The steps of cloning and the evaluation of cloninig results was shown in Figure 3.10.

```
┌─────────────────────────┐                    ⬇
│ Reamplification of 700bp │
│ band with primer CA 700  │          ┌─────────────────────────┐
│ to add restriction cut   │          │ Tansformation of ligated │
│ side                     │          │ pUC 19 into the competent│
└─────────────────────────┘          │ cell                     │
            ⬇                        └─────────────────────────┘
┌─────────────────────────┐                      ⬇
│ Extracion of band from  │          ┌─────────────────────────┐
│ agarose gel             │          │ Plating of transformant │
└─────────────────────────┘          │ E.coli cells            │
            ⬇                        └─────────────────────────┘
┌─────────────────────────┐                      ⬇
│ Restriction enzyme      │          ┌─────────────────────────┐
│ digestion of band and   │          │ Plasmid isolation from  │
│ vector                  │          │ white colonies          │
└─────────────────────────┘          └─────────────────────────┘
            ⬇                                    ⬇
┌─────────────────────────┐          ┌─────────────────────────┐
│ Ligation of band into   │          │ Visulation of plasmids on│
│ the pUC 19              │          │ agarose gel             │
└─────────────────────────┘          └─────────────────────────┘
            ⬇
┌─────────────────────────┐
│ Preparation of competent│
│ E. coli XL1 Blue MRF    │
└─────────────────────────┘
```

Figure 3.10. Strategy of cloning of RAPD-PCR product
into the pUC 19

Firstly, 700 bp amplification product was reamplifed by Primer CA 700
to add *Hind*III cut side, after agarose gel electrophoresis the band was extracted
from agarose gel by using DNA Extraction Kit of Fermentas as mentioned in

section 2.2.5. until concentrated amount of fragment was obtained. The results of extraction was shown in Figure 3.11.

The results of extraction was shown in Figure3.11.
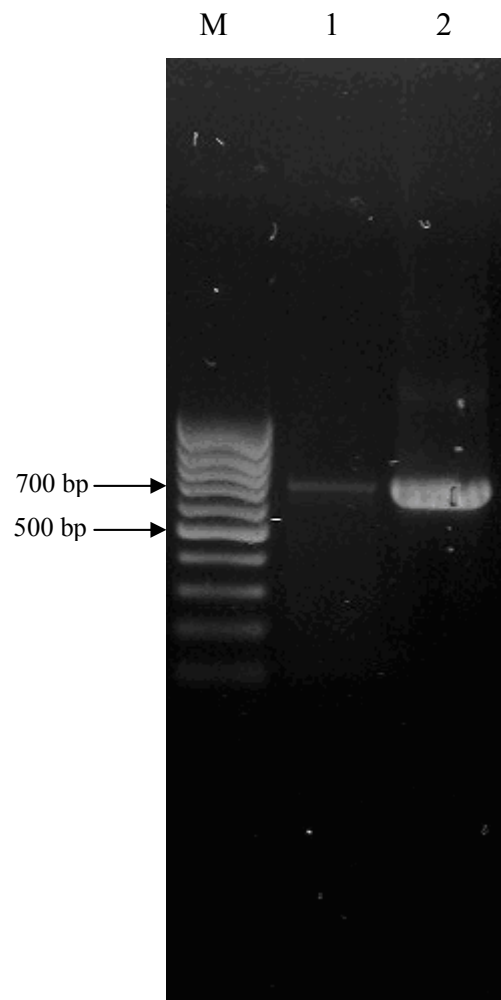
Figure 3.11. The results of extraction; **M.** 100 bp DNA Ladder;
1. First extraction result of band; **2.** Last extraction result of band which
was concentrated one used for in ligation step.

Both concentrated 700 bp band and pUC 19 vector (Appendix D) were digested with *Hind*III enzyme (2.2.6) seperatly to get cohesive ends. Digested

pUC 19 vector (Appendix D) was dephosphorylated with alkaline phosphatase to prevent self ligation by removing 5' phosphate and ligation procedure was carried out as described section 2.2.7. Ligated vector was transformed competent *E. coli XL1 Blue MRF* (Section 2.2.8.2). Transformed cells incubated overnigth at 37°C after spreading on 2YT ampicilin agar plates (Appendix B) that had been exposed with X-gal and IPTG (Appendix B). At the end of incubation time four white and two blue colonies were observed. White colonies were picked up separetly and cultivated in 2YT broth that contain 100 μg/ml ampicilin, then plasmid isolation was performed as mentioned in section 2.2.9. The results of plasmid isolation was shown in below figure (Figure 3.12.)
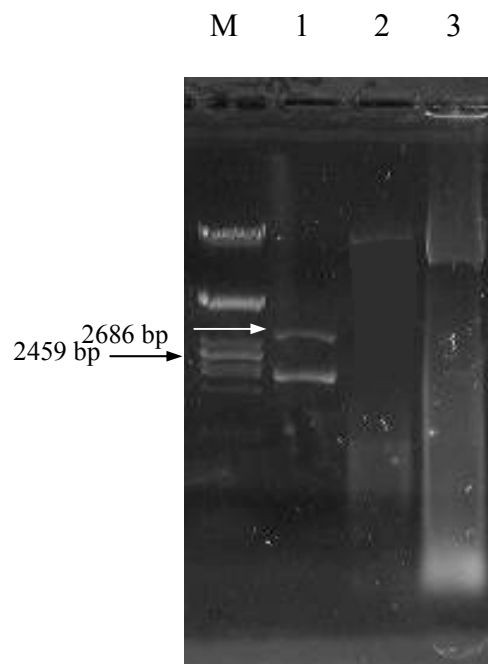
Figure 3.12. Plasmid isolation results; **M.** λDNA/*Pst*I; **1.** pUC 19 that did not contain insert used as a positif control; **2.** White Colony I; **3.** White Colony II.

Plasmid isolation was carried out many times for four white colonies by controling and adjusting the chemicals's pH values and process times. At the end of the plasmid isolation of four white colonies, no plasmids were obtained. pUC 19 which did not contain insert was transformed *E. coli XLI Blue MRF* competent cells and used as a positive control in plasmid isolation step. After that same plasmid isolation procedure was carried out for positive control, plasmid was obtained (Figure 3.12.). Therefore, the fail of the plasmid isolation procedure had low possibility. Acccording to these results, it might be possible that the insert carrying plasmids had found a homolog region on *E. coli* genom and integrated itself there.

CHAPTER 4

CONCLUSION

In this study it was aimed to find the specific DNA marker by RAPD-PCR method for identifcation and detection of *Salmonella* Typhimurium which is one of the most common reason of the food poisoning all over the world. Therefore, development of rapid and easy detection and identification method for *S.* Typhimurium is important for both food industry and human health.

In RAPD-PCR reaction , Primer 3 (RAPD 9.1), 9 bp in size, was used. It was selected according to the results of previous study which was performed by Ören in 1997. All *Salmonella* Typhimurium isolates was used in this study gave strong amplification product about 700 bp, also some of them gave additional bands at about 300 bp, 800 bp, 900 bp, 1000 bp in size.

Four *Salmonella* Typhimurium strains of NCTC (National Culture Type Collection) and Institute of Pasteur which were obtained from Refik Saydam Culture Collection used for the confirmation of the 700 bp amplification fragment of other *Salmonella* Typhimurium strains using primer 3 (RAPD 9.1). These four   *Salmonella* Typhimurium strains gave the 300 bp strong amplification band in addition to the 700 bp.

Isolates of other *Salmonella* serotypes which were given in Table 2.2 did not produce any 700 bp band. However, some of them gave about 300 bp amplification product. Moreover, this 300 bp band had not been obtained by

other different *Salmonella* serotypes, *S*. Typhi, *S*. Paratyphi B, *S*. Anatum, *S*. Strasbourg, S. Orion, *S*. Rissen, which had been used in the study of Ören (1997). So 300 bp amplification band could not be used as identification marker both for serotype *Salmonella* Typhimurium and *Salmonella* genus.

The band 700 bp in size was ligated into the vector pUC 19 to sequence. Although the cloning operation gave pozitif results by the formation of blue and white colonies, plasmid isolation process was not achived for white colonies. Therefore, sequencing of the 700 bp fragment together with plasmid DNA could not be sequenced.

As a results, 700 bp amplification product was found as a specific polymorphic region for *Salmonella* Typhimurium and this band can be used as a specific marker. Our next step will be the sequencing of this fragment by commercial firm located in USA. After sequencing process of 700 bp fragment, the location of it on the *Salmonella* Typhimurium LT2 genome will be determined. Furthermore, specific and long primer(s) and also specific probe can be designed for detection and identification of *Salmonella* Typhimurium. Both specific primer(s) and specific probe can be used in food industry for rapid detection and identification of *Salmonella* Typhimurium.

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APPENDIX A

CHEMICALS, ENZYMES AND THEIR SUPPLIERS

Agar	Oxoid
Agarose	Sigma
Ampicilin	Mustafa Nevzat İlaç San.
ATP	MBI Fermentas
Calcium Chloride	Merck
Chloroform	Merck
100 bp DNA Ladder Plus	MBI Fermentas
dNTP	MBI Fermentas
EDTA	Merck
Etidium Bromide	Sigma
Glacial Acetic Acid	MBI Fermentas
Glucose	Merck
*Hind*III restriction enzyme	MBI Fermentas
IPTG	MBI Fermentas
Maltose	Difco
Mineral Oil	Sigma
NaCl	Merck
NaOH	Merck
N,N'dimethylformamide	Sigma
Proteinase K	MBI Fermentas
Phenol:Chloroform:Isoamyl alcohol	Sigma
RNase A	Roche
SDS	Merck
Sodium Acetate	Merck

Sucrose	Merck
Taq DNA Polymerase	MBI Fermentas
T4 DNA Ligase	Sigma
Tris	Merck
Tryptic Soy Broth	Merck
Tryptic Soy Agar	Lab M
Tryptone	Difco
X gal	MBI Fermentas
Yeast extract	Merck
λDNA	MBI Fermentas
λDNA/*Hind* III	MBI Fermentas

APPENDIX B

PREPARATIONS OF GROWTH MEDIA, BUFFERS AND SOLUTIONS

1. Agarose (0.8%)

0.8 g agarose is dissolved in 100 ml TAE buffer by heating in microvave

2. DNase-free RNase

RNase A is dissolved in 0.01 M NaAc (pH = 5.2) to give a final concentration of 10 mg/ml. The solution is heated to 100°C for 15 minutes in a boiling water bath for the inactivation of DNase. It is cooled slowly to room temperature. 0.1 volume of 1 M Tris-HCl (pH = 7.5) is added until the pH of the solution is 7.0. The solution is dispensed into aliquots and stored at -20°C.

3. EDTA (0.5 M, pH 8.0)

186.1 g of disodiummethylenediaminetetraacetate is added to 800 ml of distilled water. It is stirred vigorisly on a magnetic stirrer while the pH is adjusted to 8.0 with NaOH pellets. The solution is dispend into aliquots sterilized by autoclaving.

4. Ethanol (70%, 100 ml)

70 ml ethanol is mixed with 30 ml sterile water.

5. Extraction Wash Buffer (6 ml)

150 µl Concentrated Wash Buffer (DNA Extraction Kit - Fermentas)
2.85 ml distilled water
3 ml 95 % ethanol

6. IPTG (100 mM)

0.24 g IPTG is dissolved in 10 ml H_2O, filter sterilized, dispensed into aliquots and stored at 4°C.

7. Maltose Solution 20% (w/v)

10 g maltose is disolved in 50 ml distilled water and filter sterilized.

8. $MgSO_4$ (10 mM, per Liter)

2.46 g $MgSO_4.7H_2O$ is dissolved in 1 liter distilled water. The solution is sterilized by autoclaving.

9. 3M Na Acetate pH 5.2

When 400 ml of distilled water stir in a 1 liter graduated cylinder, 408.2 g of sodium acetate is added slowly in dH_2O and during 40-60 min is stirred continuosley. Then pH is adjusted to 5.2 by adding glacial acetic acid. The final volume is adjusted to 1 liter with dH_2O. Sterilization is prepared by filtration.

10. 10% SDS

100 g of electrophoresis-grade SDS is dissolved in 900 ml of distilled water and heated to 68°C to assist dissolution, then the pH is asjusted to 7.2 by

adding a few drops of concentrated HCI. The volume is adjusted to 1 liter with water. There is no need to sterilize 10% SDS.

11. Soultion A

50 mM $CaCl_2$
10 mM Tris-HCl (pH 8.0)

12. Solution I

50 Mm Glucose
25 mM Tris-HCl (pH 8,0)
10 mM EDTA

13. Solution II

0,2 N NaOH
1% SDS

14. Solution III

3 M NaAC (pH 4,8)

15. TAE Buffer (50X per Liter)

242 g of Tris base is dissolved in 600 ml of distilled water. The pH is adjusted to 6.0 with glacial acetic acid. Then 100 ml of 0.5 M EDTA (pH 8.0) is added the volume is adjusted to 1 liter.

16. TE Buffer

10 mM Tris-HCI (pH 8.0)
1mM EDTA (pH 8.0)

17. Tris (1M, pH 8.0)

121.1 g of tris base is dissolved in 800 ml of distilled water. The pH is adjusted to 8 by adding concentrated HCI. The solution is allowed to cool to room temprature before making final adjustment to the pH. The volume is adjusted to 1 liter with water. Dispense into aliquots and sterilized by autoclaving.

18. 2YT Medium (1 liter)

16 g bacto-tryptone, 10 g yeast extract, and 5 g NaCl are dissolved in 900 ml of distilled water by shaking. The pH is adjusted to 7.0 with NaOH and the volume is adjusted to 1 liter with distilled water. Sterilization is performed by autoclaving.

19. 2YT Ampicilin Agar

16 g bacto-tryptone, 10 g yeast extract, 5 g NaCl and 15 g bacto-agar are dissolved in 900 ml of distilled water by shaking. The pH is adjusted to 7.0 with NaOH. After adjusting the volume to 1 liter with distilled water, the medium is autoclaved. 400 µl 250 mg/ml ampicilin is added when it cools to 55°C, and poured to petri dishes. The plates are covered with parafilm and stored in dark at 4°C.

20. X-gal (2%)

20 mg X-gal was dissolved in 1 ml N,N'-dimethylformamide, covered with foil and stored at -20°C.

APPENDIX C

DNA SIZE MARKERS
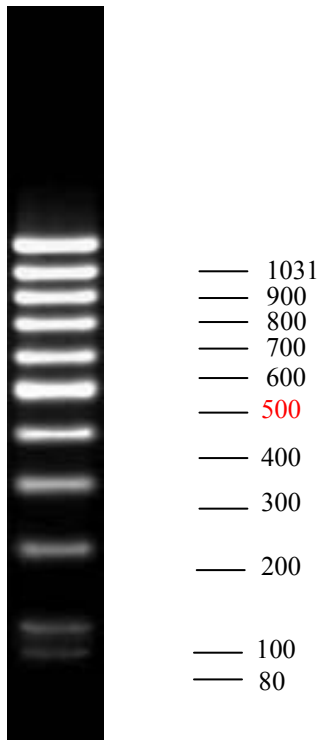
Figure C1: Gene Ruler
100bp DNA Ladder

Figure C2: GeneRuler 100bp
DNA Ladder Plus

Figure C3: λ DNA/ *Hind*III

pUC 19 VECTOR MAP

BsmB I 51
Drd I 91
Drd I 91
BsmB I 51
BstAP I 179
BsmB I 2683
BstAP I 179
EcoO109 I 2674
Nde I 183
Aat II 2617
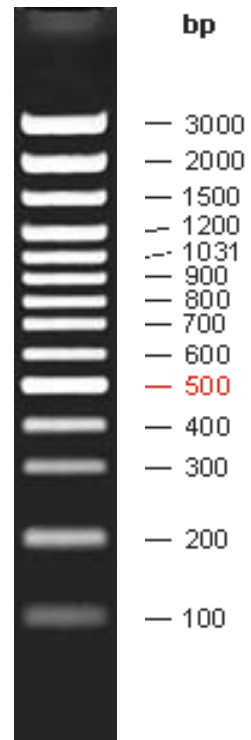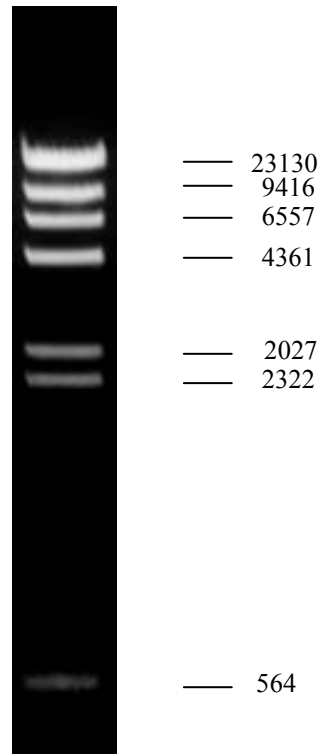Kas I - Nar I - Sfo I 235
BciV I 2542
Bgl I 245
Ssp I 2501
Fsp I 256
Pvu I 276
Pvu II 306
Acl I 2297
Bmr I 364
Xmn I 2294
BceA I 387
lacZα

Bcg I 2215
Sca I 2177
MCS

Apo I - EcoR I 396
Ban II - Sac I 402
Acc65 I - Kpn I 408
Ava I - BsoB I -
Sma I - Xma I 412
BamH I 417
Xba I 423
Acc I - Hinc II - Sal I 429
BspM I - BfuA I 433
Sbf I 434
Pst I 435
Sph I 441
Hind III 447

Pvu I 2066
Ava II 2059

BsrD I 1935
Acl I 1924
Fsp I 1919

Ap^R

Ava II 1837
Bgl I 1813
Bpm I 1784
BsrF I 1779
Bsa I 1766
BsrD I 1753
Bmr I 1744
Ahd I 1694

Pvu II 628
Tfi I 641
BsaX I 659
Sap I 683
Tfi I 781
Afl III - Pci I 806
Drd I 908

ori

BciV I 1015
BseY I 1110
AlwN I 1217
BceA I 1292

pUC19 MCS

```
              SacI     SmaI      XbaI       SbfI
     EcoRI      KpnI    BamHI      SalI PstI  SphI HindIII
  agtgAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGGcgtaatcatggtcat
       .    .    .    .    .    .    .    .    .    .    .    .    .    .
         400       410       420       430       440       450       460
  ...S  N  S  S  P  V  R  P  D  E  L  T  S  R  C  A  H  L  S  P  T  I  M  T  M
                                 ←——— lacZα translational start ———|
```

APPENDIX E

THE *Salmonella enterica* serovar Typhimurium LT2 GENOME