CHAPTER III

LITERATURE REVIEWS

Morphology and Identification of *Salmonella* species

Salmonella had been named after the scientist who discovered them, Dr. Daniel Salmon in 1884. It was classified in family Enteribacteriaceae, tribe Salmonellea, and genus Salmonella. Salmonella is facultative gram negative bacilli very in length. Colonies are most often opaque and convex. Most species except *Salmonella pullorum-gallinarum* are motile with peritrichous flagella. Salmonellae grow readily on simple media, but they almost never ferment lactose or sucrose. They form acid and sometime gas from glucose and mannose. *Salmonella* species are oxidase negative, catalase positive, indole and Voges Proskauer (VP) negative, methyl red and Simmon's citrate positive. They usually produce H_2S and urease negative. Other carbohydrates usually fermented are L-arabinose, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, trehalose and D-xylose. Some of these characteristics are used for biochemical confirmation of *Salmonella* species, as table2(2). Salmonellae are resistant to certain chemicals (eg, brilliant green, sodium tetrathionate, sodium deoxycholate) that inhibit other enteric bacteria; such compounds are therefore useful for inclusion in media to isolate salmonellae from feces(1,2).

Antigenic Structure of Salmonella species

Antigenic Structure of Salmonella species contain 3 antigen.

1. O antigen or somatic antigen is the one of cell wall component. Main substance are polysaccharide, protein and phospholipid. O antigen can resist $100^{O}C$ for two and a half hours, endure to 95% ethanol, endure to diluted acid. The reaction of O-antigen with antisera will be the granular form.

2. H antigen are flagella antigens, main component is protein. It's easy to be decomposed by alcohol, acid and temperature at $60^{O}C$. The reaction of H antigen with

antisera will be the floccules form. Almost *Salmonella* possesses 2 phases of H antigen, the first phase called specific phase and second phase called non–specific phase.

        3. Vi antigen is a special somatic antigen and belongs to the group of K antigens.It's usually capsular antigents, but a few are now known to be pili (fimbriae). These antigens are found on all encapsulated enteric organisms. In most cases, they are referred to as K antigens. By convention, however, they are called Vi antigens when they are found on salmonellae. K comes from the German word *Kapsel* and Vi is the shortened form of the word *virulence*. *Salmonella* strains with Vi antigen have an greater pathogenicity than the strains without Vi antigen(3). While salmonellae are initially detected by their biochemical characteristics, groups and species are identified by antigenic analysis. Like other *Enterobacteriaceae*, salmonellae possess several O antigens (from a total of more than 60) and different H antigens in one or both of 2 phases. Some salmonellae have capsular (k) antigens, referred to as Vi, which may interfere with agglutination by O antisera and are associated with invasiveness. Agglutination tests with absorbed antisera for different O and H antigens form the basis for serologic classification of the salmonellae. Organisms may lose H antigens and become non-motile. Loss of O antigen is associated with a change from smooth to rough colony form. Vi antigen may be lost partially or completely. Antigens may be acquired (or lost) in the process of transduction(3, 4).

Classification of Salmonella species

        The classification of salmonellae is complex. The genus *Salmonella* consists of only two species, *S. enterica*, which is divided into six subspecies: *S. enterica* subsp. *enterica*, *S.enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S.enterica* subsp. *houtenae*, and *S. enterica* subsp. *indica*, and *S. bongori* (biochemical are shown in table2)(2). This nomenclature reflects present *Salmonella* taxonomy. Serovars belonging to *S.enterica* subsp. *enterica* are disignated by a name usually related to the geographical place where this serovar was first isolated. This name is written in roman letters (not italicized) and the first letter is a capital letter. Serovars belonging to other subspecies are designated by their antigenic formulae, following the

subspecies name. More than 2,501 different serovars of *Salmonella enterica* have been identified and most of them have been described as the cause of human infections, but only a limited number of serovars are of public health importance. Most reports have mentioned *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis as the most common causes of human salmonellosis worldwide(2,5).

Table 2   Differential characters of *Salmonella* species and subspecies by biochemical test

Species	S. enterica						S. bongori
Subspecies	*enterica*	*salamae*	*arizonae*	*diarizonae*	*houtenae*	*indica*	
Characters							
Dulcitol	+	+	-	-	-	d	+
ONPG (2 h)	-	-	+	+	-	d	+
Malonate	-	+	+	+	-	-	-
Gelatinase	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	+	-	+
Culture with KCN	-	-	-	-	+	-	+
L(+)-tartrate[a]	+	-	-	-	-	-	-
Galacturonate	-	+	-	+	+	+	+
γ-glutamyltransferase	+(*)	+	-	+	+	+	+
β-glucuronidase	d	d	-	+	-	d	-
Mucate	+	+	+	-(70%)	-	+	+
Salicine	-	-	-	-	+	-	-
Lactose	-	-	-(75%)	+(75%)	-	d	-
Usual habitat	Warm-blooded animals	Cold-blooded animals and environment					

(a) = *d*-tartrate
(*) = Typhimurium d, Dublin -.+
+ = 90% or more positive reaction
- = 90% or more negative reaciton
d = different reactions given by different serovars

In 1926 the analysis procedure of O and H antigens were initiated by White and these procedures were studied deeply by Kauffman. Thus almost of references of *Salmonella* serotyping is performed according to the Kauffman – White Scheme. *Salmonella* species are also characterized by three major antigens by Kauffman–White(5). Serotyping is a definitive typing method used for epidemiological characterisation of bacteria. Serotyping of *Salmonella enterica* strains is carried out by identification of surface antigens (LPS, O-antigens) and flagella antigens (proteins, H-antigens). Most commonly strains of *Salmonella* express two phases of H- antigens but aphasic, monophasic and triphasic variants are known. The definition of the serotypes on basis on the present antigen combination is given in the "Kauffmann-White scheme"(5).

The initial serodiagnostic of *Salmonella* was performed in 1896 by using the serum of Salmonellosis patient to agglutinate with the pathogen of Salmonellosis patient. The strains which will be tested must had been tested for biochemical characterictic already, the two important tests are TSI, Urea agar, LDC, ONPG, Indole, and VP. It should be subcultured the tested strain to NA or TSA plate and incubate at $37^{O}C$ for 24 hr. before test for serological test. Detection of the O-antigen is performed by slide agglutination method. In 1997,1998 WHO Collaborating Center for Reference and Research on *Salmonella* Institute Pasteur published the report of Antigenic Formula of *Salmonella* serovar classified Genus *Salmonella* as following data Table 3 The simplified antigenic formulae of *Salmonella* serovars are listed in a document called the Kauffmann-White Scheme(6).

Table 3   Antigenic schema for *Salmonella* formulas
(Which contain O antigen and H antigen)

GROUP	SEROVAR	SOMATIC (O) ANTIGEN	FLAGELLA (H) ANTIGEN	
			phase I	phase II
O;2 (A)	*S.* Paratypni A	1,2,12	a	(1,5)
	S. Kiel	1,2,12	g,p	-
O:4 (B)	*S.* Paratyphi B	1,4,(5),12	b	1,2
	S. Derby	1,4,(5),12	f,g	(1,2)
	S. Typhimurium	1,4,(5),12	i	1,2
O:7 (C1)	*S.* Paratyphi C	6,7,(Vi)	c	1,5
	S. Schwarengrund	**1,4,(5),12**	**d**	**1,7**
	S. Livingstone	6,7,14	d	1,w
	S. Virchow	6,7	r	1,2
	S. Infantis	6,7,14	r	1,5
O:8 (C2-C3)	*S.* Newport	6,8,20	e,h	$1,2:[z_{67}]$
	S. Brunei	8,20	y	1,5
O:9 (D1)	*S.* Typhi	9,12, (Vi)	d	-
	S. Javiana	1,9,12	l,z_{28}	1,5
	S. Gallinarum	1,9,12	-	-
O:3,10 (E1)	*S.* Anatum	3,10,(15), (15,38)	e,h	1,6
	S. Weltevreden	3,10,(15)	r	z_6
	S. Lexington	3,10,(15),(15,34)	z_{10}	1,5
	*S.*Ratchaburi	3,10	z_{35}	1,6
O:11 (F)	*S.* Aberdeen	11	i	1,2
O:13 (G)	*S.* Poona	1,13,22	z.	1,6

Pathogenesis and clinical findings of Salmonella species

Many persons may contract salmonellosis many times in his or her life and not always recognize it. Most persons infected with Salmonella develop diarrhea, fever, and abdominal cramps 12 to 72 hours after infection. The illness usually lasts 4 to 7 days, and most persons recover without treatment. However, in some persons the diarrhea may be so severe that the patient needs to be hospitalized. In these patients, the Salmonella infection may spread from the intestines to the blood stream, and then to other body sites and can cause death unless the person is treated promptly with antibiotics. The elderly, infants, and those with impaired immune systems are more likely to have a severe

illness(7). Many people ill with salmonellosis recover without treatment and many never see a doctoctor. However, salmonella infections can be life-threatening especially for the very young, the elderly, and for persons with impaired immune systems (8). Persons with diarrhea usually recover completely, although it may be several months before their bondel habits are entirely normal. A small number of persons who are infected with salmonella will develop pains in their joints, irritation of the eyes, and painful urination. This is called Reiter's syndrome. It can last for months or years and can load to chronic arthritis that is difficult to treat. Most cases of salmonellosis are not treated with antibiotics but fluids are recommended to prevent dehydration because the diarrhea that flushes bacteria out of the body drains a great deal of liquid. Pain relievers and fever reducers may make the person more comfortable(9, 10). In fact, antibiotics may prolong the period during which the person can infect others. Also, antibiotics actually may bring on salmonellosis symptoms by upsetting the bacterial balance in the intestines. Antibiotics sometimes are prescribed for infants, the chronically ill and the eiderly to prevent salmonella-triggered local infections or bacterimia. Antibiotics also are needed when the bacteria cause meningitis or infections of the blood stream. People are far more likely to contract salmonellosis at home than in a restaurant, so be sure to handle food safely(11). The organisms almost always enter via the oral route, usually with contaminated food or drink. The mean infective dose to produce clinical or subclinical infection in human is 10^5-10^8 salmonellae. After *Salmonella* is eaten it passes through the stomach to the intestine. Here, it binds to the wall of the intestine, and through some special proteins that it makes in response to the particular conditions in the intestine it actually penetrates the barrier between us and the outside. Once it has gained access to our insides, it is taken to the liver or spleen. For most other bacteria, this journey would kill them, however *Salmonella* has evolved mechanisms to prevent our immune system from doing its job efficiently. In the liver, the *Salmonella* can grow again, and be released back into the intestine. Of course, not all of the *Salmonella* pass through the intestinal wall, and many of them are expelled from the intestine in the diarrhea. In regions with poor sanitation, these bacteria can than survive in the soil or in rivers and infect the next person. The infection is primarily caused by improper handling and digestion of uncooked food and a large number of different food animal sources have been identified

as reservoir of the bacteria and direct person to person contact have also been
implicated(12), (13).
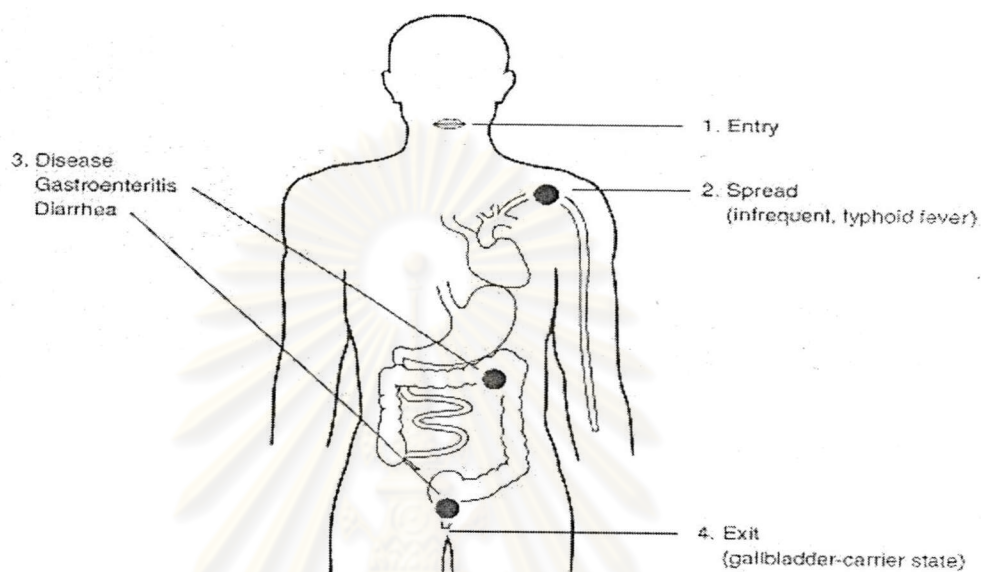
Figure 2   Routes of infection of *Salmonella enterica*

Among the host factors that contribute to resistance to Salmonellae in fection are
gastric acidity, normal intestinal microbial flora, and local intestinal immunity.
Salmonellae produce 3 main types of disease in human, but mixed forms are frequent.

1. The "Enteric fever" (Typhoid Fever): This syndrome is produced mainly by
Salmonella typhi, Salmonella paratyphi A, and Salmonella schottmulleri. The ingested
salmonellae reach the small intestine, from which they enter the lymphatics and then the
bloodstream. They are carried by blood to many organs, including the intetine. The
organisms multiply in intastinal lymphoid tissue and are excreted in stools. After an
incubation period of 10-14 days, fever, malaise, headache, constipation, bradycardia, and
myalgia occur. The fever rises to a high plateau, and the spleen and liver become
enlarged. Rose spots are seen briefly in rare cases. The white blood cell count is normal
or low. In the preantibiotic era, the chief complications of enteric fever were intestinal

hemorrhage and perforation, and the mortality rate was 10-15%. Treatment with chloramphenicol or ampicillin has reduced the mortality rate to less than 1%. Occasional *Salmonella typhi* strains resistant to these drugs have responded to trimethoprim-sulfamethoxazole. The principal lesions are hyperplasia and necrosis of lymphoid tissue, hepatitis, focal necrosis of the liver, and inflammation of the gallbladder, periosteum, lungs, and other organs.

2. Bacteremia With focal Lesions: This is associated commonly with *Salmonella choleraesuis* but may be caused by any *Salmonella* serotype. Following oral infection, there is early invasion of the bloodstream (with possible focal lesions in lungs, bones, meninges, etc), but intestinal manifestations are often absent. Blood cultures are positive.

3. Enterocolitis(Formerly "Gastroenteritis"): This is the most common manifestation of *Salmonella* infection. Eight to 48 hours after ingestion of salmonellae, there is nausea, headache, vomiting, and profuse diarrhea, with few leukocytes in the stools. Low grade fever is common, but the episode usually resolves in 2-3 days. Inflammatory lesions of the small and large intestine are present. Bacteremia is rare(2-4%) except in immunodeficient persons. Blood cultures are usually negative, but stool cultures are positive for salmonellae and may remain positive for several weeks after clinical recovery(14, 15).

Treatment of Salmonellosis

While enteric fevers and bacteremias with focal lesions require antimicrobial treatment, the vast majority of cases of enterocolitis do not. In enterocolitis, clinical symptoms and excretion of the salmonellae may be prolonged by antimicrobial therapy. In severe diarrhea, replacement of fluids and electrolytes is essential. Antimicrobial therapy is with chloramphenicol, ampicillin, or trimethoprim-sulfamethoxazole. Multiple drug resistance transmitted genetically by plasmids among enteric bacteria is a problem in *Salmonellae* infections. As many as 25% of salmonellae are resistant to ampicillin and 5% are resistant to chloramphenicol; resistance to trimethoprim-sulfamethoxazole is also increasing. In most carriers, the organisms persist in the gallbladder(particularly if gallstones are present) and in the biliary tract. Some chronic carriers have been cured by

ampicillin alone, but in most cases cholecysectomy must be combined with drug treatment(14).

Epidemiology of *Salmonella* species

Salmonellosis is a major cause of illness in Thailand and other parts of the world, an estimated 40,000 cases of salmonellosis in the United States each year, in Illinois about 1,500 to 2,500 cases of this foodborne illness are reported each year, and in Thailand about 45,192-632,684 cases / year(1). The feces of persons who have unsuspected subclinical disease or carriers are a more important source of contamination than frank clinical case that are promptly isolated, eg, when carriers working as food handlers are "shedding" organisms. Many animals, including cattle, rodents, and fowl, are naturally infected with a variety of salmonellae and have the bacteria in their tissues(meat), excreta, or eggs. The incidence of typhoid fever has decreased, but the incidence of other *Salmonella* infections has increased markedly in the USA. The problem is aggravated by the widespread use of animal feeds containing antimicrobial that favor the proliferation of drug-resistant salmonellae and their potential transmission to humans(14).

A. **Carriers**: After manifest or subclinical infection, some individuals continue to harbor salmonellae in their tissues for variable lengths of time (convalescent carriers or healthy permanent carriers). Three percent of survivors of typhoid become permanent carriers, harboring the organisms in the gallbladder, biliary tract, or rarely, the intestine or urinary tract.

B. **Sources of infection**: The sources of infection are food and drink that have been contaminated with salmonellae. The following sources are important:

1. Water – Contamination with feces often results in explosive epidemics.

2. Milk and other dairy products(ice cream, cheese, custard) – Contamination with feces is due to inadequate pasteurization or improper handling. Limited outbreaks are traceable to the source of supply.

3. Shellfish – From contaminated water.

4. Dried or frozen eggs – From infected fowl or contaminated during processing.

5. Meats and meat products – From infected animals (poultry) or contaminated with feces by rodents or humans.

6. "Recreation" drugs.

7. Animal dyes – Dyes (eg, carmine) used in drugs, foods, and cosmetics.

8. Household pets – Turtles, dogs, cats, etc.

Prevention and Control of Salmonellosis

Sanitary measures must be taken to prevent contamination of food and water by rodents or other animals that excrete salmonellae. Infected poultry, meats, and eggs must be thoroughly cooked. Carriers must not be allowed to work as food handlers and should observe strict hygienic precautions. Two injections of acetone-killed bacterial suspensions of S. typhi, followed by a booster injection some months later, give partial resistance to small infectious inocula of typhoid bacilli but not to large ones. Oral administration of a live avirulent mutant strain of S typhi has given significant protection on areas of high endemicity. Vaccines against other salmonellae giveless protection and are not recommended(14).

Salmonella Schwarzengrund

Salmonella Schwarzengrund is one of a *Salmonella* that has only been isolated sporadically from infections in humans and from different animal sources. *S.* Schwarzengrund has also been found in chicken in other studies, suggesting that poultry could be the most common reservoir (5).

Chiu et.al. (1999) studied predictors for extraintestinal infection(EII) of non-typhoidal Salmonellosis in patients without AIDS. A study of 398 patients with cultured positive for non-typhoidal *Salmonella* seen at Chang Gung Memorial Hospital and Chang Gung Children's Hospital between November 1993 and October 1994 showed that *Salmonella* schwarzengrund was the commonest causes of EII among those serotypes

usually causing gastroenteritis. Pre-existing underlying disease, particularly immunosuppressive disease, was the most important risk factor that may have predisposed adult patients to have EII. Old age (> or = 60 years) and isolation of invasive serotypes were also frequently associated with EII in adult patients. The characteristics of paediatric patients with a high probability of having EII were: < 3 years of age, abnormal blood test results (a leucocyte count > or = 15,000/mm3 or < 5000/mm3, immature leucocytes > or = 10% of total leucocytes, and a C-reactive protein concentration > or = 50 mg/l); and isolation of invasive serotypes. This information can be an aid to early diagnosis and treatment of EII caused by non-typhoidal Salmonella (16). The report of reactive arthritis dued to *Salmonella* Schwarzengrund in a patient with asymtomatioc spondylitis that haven't occured before. The patient had been hospitalized in the Philippines and had probably acquired the infection there in 1985 had been reported by Gloding and Robertson(17). One outbreak of 125 cases of foodborne salmonellosis, in

the United States of America (USA), resulted from cross-contaminated food items served at a picnic and a smorgasbord had been reported by Levy et.al. (1975). One hundred and twenty-five of 173 people who ate at a picnic and/or a smorgasbord prepared by a bar-restaurant developed diarrhea, abdominal cramps, and other symptoms 23 hours (median time) later. Eleven were hospitalized. Stool cultures from 18 ill individuals grew *Salmonella* Infantis, *Salmonella* Agona, and S*almonella* Schwarzengrund. Stool cultures from 5 of 8 restaurant employees were found *Salmonella* Infantis or *Salmonella* Agona. Cultures of remaining foods and food-contact surfaces were negative. Food-specific attack rates, based on interviews with 121 eaters, implicated potato salad and chicken dressing as vehicles of transmission, both likely contaminated when prepared in pans that shortly before contained uncooked, chicken pieces suspected to have harbored salmonellae. Chicken were eventually traced to 3 farms where feed samples were found to contain *Salmonella* Typhimurium and *Salmonella* Cubana, raising the possibility that other feed samples may have contained the serotypes responsible for the outbreak. The main control measure was temporarily closing the food service, which was to have catered a large church picnic the next day. The outbreak had an economic impact estimated at $28,733 (18). Another report of Salmonellosis outbreak in human caused from food of animal origin was reported by Lipson and Meikle (1977). A powdered

preparation of porcine pancreatin contaminated with *Salmonella* Schwarzengrund and *Salmonella* Eimsbuettel resulted in the infection of at least 31 % of one group of paediatric patients with cystic fibrosis. The pancreatin contained very small numbers of *Salmonellae*, the infecting dose in at least one child being less than 44 organisms. More stringent bacteriological standards are needed for pharmaceuticals and foods used by paediatric patients (19).

An epidemiological survey of antibiotic resistance of *Salmonella* in market-age swine in 1999 in the USA by Harvey et.al. This study conducted a survey of antibiotic resistance in *Salmonella* isolated from market-age swine at five different farms. Samples were taken from the lymph nodes and cecal contents at the time of slaughter. Of the 559 *Salmonella* isolates recovered, 420 were sent to the National Veterinary Services Laboratory for serotyping. Resistance patterns were determined by disk diffusion using thirteen antibiotics, and 320 isolates were analyzed. Resistance was observed to ampicillin, chloramphenicol, chlortetracycline, nitrofurantoin, penicillin G, streptomycin and sulfisoxazole. Multi-drug resistance (resistance to two or more antibiotics) was observed in 183 isolates (57.2 %) with 34 of these isolates (18.6 %) resistant to three antibiotics and 26 (8.1 %) resistant to four antibiotics. The most common three drugs resistance pattern consisted of chlortetracycline, penicillin G and streptomycin (37.3 %). A significant difference was observed between serotypes and between somatic serogroups in their antibiotic resistance patterns. Variation also was observed between farms and between seasons in which sampling occurred. As a step in understanding the connection between antibiotic use in agriculture and medicine and emergence of antibiotic resistant bacteria, programs that monitor the levels of antibiotic resistance must be continued. In animal production, where sub-therapeutic administration of antibiotics is extremely common, continued surveillance is especially important (20).

Olsen et.al. (2001) had reported the first recognized outbreak of fluoroquinolone-resistant *Salmonella* infection in the USA., which occurred in two nursing homes and one hospital in Oregon. By interviewed medical staff and reviewed patients' charts and death certificates. In Nursing Home A conducted a case-control study. Patients were defined as residents of the nursing home from whom fluoroquinolone-resistant *Salmonella enterica* serotype Schwarzengrund was isolated between February 1996 and December 1998.

Controls were residents with similar medical conditions whose cultures did not yield *Salmonella*. This study compared isolates using pulsed-field gel electrophoresis and sequence analysis and reviewed pharmacy records to compare the use of fluoroquinolone among several nursing homes. Eleven patients with fluoroquinolone-resistant salmonellosis were identified at two nursing homes. The index patient had been hospitalized in the Philippines and had probably acquired the infection there. Transmission was probably direct (from patient to patient) or through contact with contaminated surfaces. Treatment with fluoroquinolones during the six months before a culture was obtained was associated with a significant risk of Salmonella infection (4 of 5 patients had taken fluoroquinolones, as compared with 2 of 13 controls; odds ratio, 22.0; 95 percent confidence interval, 1.06 to 1177). The patients were not significantly more likely than the controls to have taken other antibiotics. More fluoroquinolones were used at Nursing Home A than at similar nursing homes in Oregon. The isolates from the outbreak had similar patterns on pulsed-field gel electrophoresis and the same gyrA mutations. The isolates from the outbreak were also similar to the only previous isolate of fluoroquinolone-resistant *Salmonella* in the United States, which came from a patient in New York who had been transferred from a hospital in the Philippines. From this acknowledge describe a prolonged nosocomial outbreak of infection with fluoroquinolone-resistant *Salmonella* enterica serotype Schwarzengrund. More such outbreaks are likely in institutional settings, particularly those in which there is heavy use of antimicrobial agents (21).

The prevalence of *Salmonella* Enteritidis and other *Salmonella* spp. among Canadian registered commercial chicken broiler flocks in 1991 had been studied by Poppe et.al. A nation-wide survey was conducted to estimate the prevalence of Salmonella enteritidis and other *Salmonella* among Canadian commercial broiler flocks. The environmental (litter and/or water) samples from randomly selected flocks were found contaminated with *Salmonella* 76.9%. which the most prevalent serovars were *Salmonella* Hadar (33.3%), *Salmonella* Infantis (8.8%), and *Salmonella* Schwarzengrund(7.1%). While the faecal and eggbelt samples from randomly selected flocks were found contaminated with *Salmonella* 52.9%. Thirty-five different Salmonella serovars were which the most prevalent serovars were *Salmonella* Heidelberg (20 %),

Salmonella Infantis (6.1 %), *Salmonella* Hadar (5.8 %), and *Salmonella* Schwarzengrund (5.1 %)(5). Another study of *Salmonella* contamination in environment had been performed in Germany by Wicke et.al. (1986). *Salmonella* investigations were carried out in a total of 54 water samples taken from the sewage system of the town Kiel. There were able to isolte 6,181 strains of *Salmonella* from the sewage samples which the most predominant serovars were *Salmonella* Typhimurium, *Salmonella* Panama, *Salmonella* Schwarzengrund and *Salmonella* Hadar (22).

Salmonella Schwarzengrund was one of the invasive *Salmonella* infection reported in the USA during 1996-1999. Five-hundreds and fourty patients with *Salmonella* Schwarzengrund invasive infections 386 (71%) were hospitalized and 29 (5%) died; 13 (45%) of the deaths were among persons aged equal to or greater than 60 years. Invasive Salmonella infections are a substantial health problem in the United States and contribute to hospitalizations and deaths (23).

The method to study epidemiological typing of bacteria

Typing of bacteria isolates can be used to identify the potential sources of different bacteria stains, to evaluate the role of human carries, and to differentiate epidemic from endemic stains. Reducing the number of bacteria infections by detecting and eradicating the sources of the organisms or by interrupting their path of transmission to the patients is an important goal and requires the ability to distinguish individual stain (24). Improved typing systems are important for implementing appropriate infection control measures and for the clinical management of bacteria infections, particularly in evaluating the efficacy of therapy for infected or colonized patients. The ideal typing system should be rapid, inexpensive, technically simple, and readily available (25).

Each typing system should have 4 criteria : type ability, reproducibility, discriminatory power, ease of use.

1) Typeability refers to the ability of the test to provide an unambiguous result for each isolate examined; nontypable isolates are those that produce a null or ambiguous result.

2) Reproducibility refers to the ability of a technique to produce the same result when a strain is tested repeatedly.

3) Discriminatory between defines the ability if the test to discriminate between unrelated isolates. This discrimination is important because some typing systems tend to group organisms into a few broad groups, while others divide collections of isolates into many small clusters, often subdividing groups of isolates that are tightly linked by epidemiological data.

4) Ease of interpretation and also are also key issues for many techniques. the greater the expertise that is required to discern differences between strains, the less likely the techniques is to be readily accepted by clinical microbiologists who generally lack such expertise(26).

However, the choice of the right typing method is often difficult because of inherent theoretical and technical limitation(27). Epidemiological typing systems for differentiating among strains of bacteria are phenotyping and genotyping.

1. Phenotyping

Phenotyping characterstics, such as antibiogram and phage typing, have been widely used in epidemiological studies of bacteria. However, because these methods detect phenotyping variation that is difficult to relate to allelic variation at specific gene loci, they have not provided the information on frequencies of alleles(28). Isolates of the same genotyoes can exhibit either positive or negative phenotype. Details of each main typing methods including advantages and disadvantages ate described briefly in the following section.

1.1 Antimicrobial susceptibility patterns

In epidemiological investigations, antibiogram typing is a simplest and traditional typing method used to distinguish between individual strains. It is readily available, easy to perform, and relatively inexpensive. However, one drawback of the method is that markers of antibiotics resistance are often carried by labile or movable

genetic elements (e.g., plasmids or transposons) whose selection of expression may depend on environment condition. They may be gained or lost over time(29). Antibiogram typing worked reasonably well, but only when zone diameters and not the categorical interpretations of susceptible, intermediate, or resistant were used s strain markers(26). Thus, antibiogram typing is considered to have poor discriminatory power and is used by microbiologists only in the first instance for rapid screening of the similarities between different clinical isolates.

Blanc et al. in 1997 (29) reported that the good results of antibiogram typing in their study were probably due to these following reason : i) the usual qualitative antibiogram analysis was refined by adding quantitative measurement of inhibition zone around antibiotic disks, ii) the five antibiotics used (i.e. eruthromycin, clindamycin, co – trimoxazole, gentamycin, and ciprofloxacin)had been selected because they were mostly chromosomal markers which were unlinked and varied among the bacteria strains isolates in our hospital ; and iii) the analysis was restricted to isolates of a well-defined epidemiological setting. Although some genetically inrelated strains show great similarity by antibiogram typing, this appeared to be relatively infrequent and may be offset by the speed, availability, and the relatively high discriminatory power of this technique. The mthod could easily be adapted to the epidemiological setting of the other gospitals bu changing or adding antibiotics to increase the technique's potential for discrimination and redefining the cut off value to fit the particular hospital and laboratory setting.

Although antibiogram is valuable, especially in routine laboratories, as a first – line screening method to determine strain relatedness. It mat allow quick and early recognition of a previously defined epidemic strain in a particular hospital setting.(30)

1.2 Bacteriophage typing

This method has been used widely for typing *Staphylococcus aureus* for many years. However, its limitation are clearly recognized, a substantial number of S.aureus isolates can not be typed by phages (29, 30). This technique also requires maintenance of a large number of phage stocks and propagating strains, laborious, and it characterized isolates on the basis of a phenotypic marker that has poor reproducibility. Whatever

bacteriophage typing is not a cost-effective method of typing S. aureus for most clinical laboratories, particularly since other available methods can be used to type a broader range of other microorganisms(26).

1.3 Capsular typing

Capsular typing has only a very low power of discriminating between S.aureus strains. This method is not detected individual strains or clonal population(28).

1.4 Electrophoretic protein typing and immunoblots

The proteins and other bacterial products expressed by bacteria can be analyzed using the procedure based on sodium dodesyl sulfatepolacrylamide gel electrophosis (SDS-PAGE). The materials in the gel can be detected directly by staining or indirectly be preparation of immunobolts. Additionally, the protein can be also labeled by radioisotope and detected by autoradiography. All strains can be typeable by these approaches, which typically identify variation among independent isolates and provide good discriminatory potential. However, due to the complexity of the patterns resolved, comparison among multiple strains can be difficult and significance of small differences in the method for extracting the material from the organisms, the methods detecting proteins or the choice of antibody for developing immunoblots.

1.5 Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MLEE) has shown to be the powerful tool for population genetic studies of various bacteria, including S.aureus.(31) Isolates are characterized by the relative electrophoretic mobilities of a large number of water-soluble cellular enzymes. The enzyme binding patterns generated by starch gel electrophoresis during MLEE were easy to interpret, but the overall analysis required application of sophisticated algorithms and computer software not readily available in most laboratories. This makes this technique inaccessible to most clinical laboratories(27).

2. Genotyping (DNA-based study)

DNA – based typing of bacterial strains is based on the principle that epidemiologically related bacterial isolates have genetic feature that distinguishes them from other epidemiologically unrelated strains. Because bacteria strains probably originate from a single clone or at least a few strain types, the techniques used to type bacteria must be particularly discriminatory. Suitable typing procedures should combine typeability, discriminatory potential, easily interpretable results and practicality(32).

Molecular typing systems can identify different strains within a species, generating data useful for taxonomic or epidemiological purposes. A frequently observed fault of typing systems is their lack of reproducibility: most typing systems do not provide a definitive strain identification, which is usually due to the variability of the technique and the lack of large databases containing fragment patterns from a wide variety of organisms to which unknowns can be compared(33).

2.1 Plasmid analysis

Plasmid analysis was the first DNA-based method to be applied to S.aureus and it has been used in a number of outbreak investigations this technique have been reported to be superior to phage typing of bacteria. This technique offers only moderate reproducibility and gas limitations that some strains, which unrelated to the outbreak may have the epidemic profiles. The potential usefulness of plasmid digestion profiles to bacterial epidemiology depends upon the degree to which four requirements ate satisfied (34).

i) The bacterial strains under study must contain plasmids

ii) Plasmids must be sufficiently diverse so that independent isolates, not recently derived from a common progenitor, are likely to carry distinctly different plasmid DNA.

iii) Differences between plasmids must be distinguishable by the fragments that appear after restriction endonuclease digestion.

iv) Method for plasmid profiling must be rapid, reproducible, inexpensive, and accessible to clinical laboratories.

v) This technique also has sever drawback as following:

    1) Not all pathogenic strains gave plasmids,

    2) Plasmids are exchange and lost readily

    3) Plasmids are subject to rearrangements

    4) Some specific plasmid isolation protocols may limit the size of recoverable plasmids and thereby preclude the isolation of largeR-factors.

2.2 Restriction enzyme analysis of chromosomal DNA

In the conventional restriction enzyme analysis, chromosomal DNA is digested with restriction enzymes which have frequent recognition site, and gains a large numbers of relative small restriction fragments. Then analyze the digested profiles by constant field agarose gel electrophoresis, which separates the restriction fragment by size. The different profiles of the bacterial isolates from the same species can occur because of the nucleotide sequence variations that alters the distribution of the restriction sites along the chromosome. The results show a large number of fragments that are close together on an agarose gel and must be difficult to analyze.

2.3 Southern blot analysis of chromosomal DNA

The DNA restriction fragments separated by electrophoresis can be transferred from agarose gel onto nitrocellulose membrane which the DNA binds tightly. This membrane can htbridized with specific DNA probe to identify the restriction fragments carrying sequences complementary to the specific probe. The variation in the number and size of such fragments are referred to as restricted fragment length polymorphisms (RFLPs) and reflected variations in the nucleotide sequences within or flanking the loci of interest. All strains carrying these sequences of interest can be typed by this technique(35).

Ribotyping is based on Southern Blot analysis of the RFLPs associated with the ribosomal RNA operons, which carry by all bacteria. Therefore all strains are typeable. The random DNA mutations after the distribution of restriction sites within and adjacent to the loci being probed. Such changes are relatively infrequent within the bacterial rDNA operons the most appropriate criterion for interpreting robotype banding banding patterns remains unresolved(26).

2.4 Polymerase Chain Reaction (PCR)

PCR becomes more widespread in clinical laboratories. This technique is highly sensitive to detect outbreak-related strains, however, it failed to exclude the nonrelated isolates from the cluster. The PCR assay appears to be more reliable than routine susceptibility testing and as accurate as DNA probe hybridization. The PCR assay is simpler, more rapid test for detection of the bacteria – PBP gene than Southern Blote hybridization. PCR assay could be particularly useful in outbreaks where rapid control of bacteria is required and in countries where high incidence of clonal bacteria strain are observed in hospitals. But their sensitivity is not enough. Even PCR – based typing methods are clearly faster and easier to perform, but this technique has less discriminatory power than Pulsedfield Gel Electrophoresis(32).

2.5 Pulsed – field Gel Electrophoresis (PFGE)

In recent pears, there has been much interest in chromosomal genotyping of bacteria. The method is attractive because of its potential to type all organisms. However the difficulties in interpretation of the results of digestion with restriction enzyme which cut at frequent sites led to examination of alternative approach. One of these has been to prepare chromosomal DNA in agarose blocks, which minimize, shearing, and then to digest it with rare cutters. The large fragments are pulsed electrically into agarose (36).

The development of PFGE enabled large fragments of DNA (>9000 kb) to be separated. Varistions of this technique include contour-clamp homogenous electric field and field inversion gel electrophoresis. PFGE is multidirectional, continually changing

the location of the positive charge. The DNA molecules respond by continuously reorienting hire directions of migration through the agarose gel. In addition, electrical pulses of different favor the reorientation of different sized DNA molecules.

PFGE demonstrated the best discrimination, although this method is the most time consuming when compares to the other typing method, taking excess of 48 h to obtain result. Several publication have already confirmed the value of PFGE in S.aureus typing, reproducibility and discrimination between unrelated clones(24, 26, 28).

PFGE has been proposed as a good typing technique for epidemiological studies of most species. Profiles types generated by this technique appear stable and reproducible. Chromosomal stability makes this technique suitable for the ling – term follow – up of epidemic strains of bacteria(24).

Two main advantages of using PFGE are : i) it had universal S.aureus strain typibg ability and ii) it was better than other tuping techniques at distinguishing epidemiologically related strains from unrelated strains. Because of these advantages, at present the Center of Diseasse Control and Prevention (CDC) uses PFGE in epidemiologic investigation of S.aureus(34).

Antimicrobial susceptibility test

Since their discovery during the 20th century, antimicrobial agents (antibiotics and related medicinal drugs) have substantially reduced the threat posed by infectious diseases. The use of these "wonder drugs", combined with improvements in sanitation, housing, and nutrition, and the advent of widespread immunization programmes , has led to a dramatic drop in deaths from diseases that were previously widespread, untreatable .and frequently fatal. Over the years, antimicrobials have saved the lives and eased the suffering of millions of people. By helping to bring many serious infectious diseases under control, these drugs have also contributed to the major gains in life expectancy experienced during the latter part of the previous century. These gains are now seriously jeopardized by another recent development: the emergence and spread of microbes that are resistant to cheap and effective first-choice, or "first-line" drugs. The bacterial infections which contribute most to human disease are also those in which emerging

antimicrobial resistance is most evident: diarrhoeal diseases, respiratory tract infections, meningitis, sexually transmitted infections, and hospital-acquired infections(37). The development of resistance to drugs commonly used o treat malaria is of particular concern, as is the emerging resistance to anti-HIV drugs. The consequences are severe. Infections caused by resistant microbes fail to respond to treatment, resulting in prolonged illness and greater risk of death(38). Treatment failures also lead to longer periods of infectivity, which increase the numbers of infected people moving in the community and thus expose the general population to the risk of contracting a resistant strain of infection. When infections become resistant to first-line antimicrobials treatment has to be switched to second- or third-line drugs, which are nearly always much more expensive and sometimes more toxic as well. For example, the drugs needed to treat multidrug-resistant form of tuberculosis are over 100 times more expensive than the first-line drugs used to treat non-resistant forms. In many countries, the high cost of such replacement drugs is prohibitive, with the result that some diseases can no longer be treated in areas where resistance to first-line drugs is widespread. Most alarming of all are diseases where resistance is developing for virtually all currently available drugs, thus raising the spectre of a post-antibiotic era. Even if the pharmaceutical industry were to step up efforts to develop new replacement drugs immediately, current trends suggest that some diseases will have no effective therapies within the nest ten years. Microbes (the collective term for bacteria, fungi, parasites, and viruses) cause infectious diseases, and antimicrobial agents, such as penicillin, streptomycin, and more than 150 others, gave been developed to combat the spread and severity of many of these diseases. Resistance to antimicrobials is a natural biological phenomenon that can be amplified or accelerated by a variety of factors, including human practices. The use of an antimicrobial for any infection, real or feared, in any dose and over any time period, forces microbes to either adapt or die in a phenomenon known as **"selective pressure"**(39). The microbes which adapt and survive carry genes for resistance, which can be passes on. Bacteria are particularly efficient at efficient at enhancing the effects of resistance, not only because of their ability to multiply very rapidly but also because they can transfer their resistance genes, which are passed on when the bacteria replicate. In the medical setting, such resistant microbes will not be killed by an antimicrobial agent during a standard course of

treatment. Resistant bacteria through "conjugation", whereby plasmids carrying the genes jump from one organism to another. Resistant to a single drug can thus spread rapidly through a bacteria population. When antimicrobials are used incorrectly – for too short a time, at too low a dose, at inadequate potency, or for the wring disease – the likelihood that bacteria and other microbes will adapt and replicate rather than be killed is greatly enhanced. Since early 1950, farmers have administered low doses of penicillin and tetracycline to cows, chicken and pigs to prevent infection and promote growth. As a result, the bacteria in these animals develop a resistance to the drugs. When these drugs are used to treat infections in humans who have eaten meat from treated animals, the drugs are not as effective as they might be. The U.S. Centers for Disease Control and Prevention estimates that between 20 percent and 30 percent of all *Salmonella* cases involve bacteria resistant to antibiotics. Much evidence supports the view that the total consumption of antimicrobials is the critical factor in selecting resistance. Paradoxically, underused through lack if access, inadequate dosing, poor adherence, and substandard antimicrobials may play as important a role as overuse. For these reasons, improving use is a priority if the emergence and spread of resistance are to be controlled. In the past, medicine and science were able to stay ahead of this natural phenomenon through the discovery of potent new classes of antimicrobials, a process that flourished from 1930-1970 and has since slowed to a virtual standstill, partly because of misplaced confidence that infectious diseases had been conquered, at least in the industrialized world. In just the past few decades, the development of resistant microbes has been greatly accelerated by several concurrent trends. These have worked to increase the number of infections and thus expand both the need for antimicrobials and the opportunities for their misuse (40, 41). Antimicrobial resistance is an ever-increasing problem in hospitals, especially in intensive care units. The problem is so severe that some authorities believe that we are entering the "post-antibiotic era" where widespread bacterial resistance will render most antibiotics ineffective.No-one is sure, but the principle cause appears to be inappropriate and profligate use of antibiotics, especially in the hospital environment. Several studies have shown widespread misuse of antibiotics. Another major problem is spread of infecting organisms from patient to patient. This is usually done by the nurses, doctors

and others caring for the patient. The major method of spread is on contaminated hands(25).

Antibiotics are chemicals that inactivate or damage bacteria. Most are naturally produced by bacteria and fungi, others are man-made but have the same effect. The term antibiotic is popularly used to describe this whole range of chemicals. Strictly, it should only apply to the naturally derived chemical substances: we will use this popular terminology here. Antibiotics are administered to animals to kill bacteria that are causing infections (therapeutic and prophylactic antibiotics). Bacteria acquire genes for resistance in three ways(39).

1. In spontaneous mutation, bacterial DNA may change spontaneously, as indicated by the starburst. Drug-resistant tuberculosis arises this way.

2. In a form of microbial sex called transformation, one bacterium may take up DNA from another. Penicillin-resistant gonorrhea results from transformation.

3. Most frightening, however, is resistance acquired from a small circle of DNA called a plasmid. Plasmids can flit between bacteria of various types -- they generally must be touching -- and carry multiple resistance. In 1968, 12,500 Guatemalans died in an epidemic of Shigella diarrhea, caused by a microbe harboring a plasmid that conferred resistances to four antibiotics. But bacteria do something much more clever than just mutating. That's chancy, so bacteria prefer to share biochemical secrets -- resistance genes -- that enable them to resist or destroy antibiotics.

The resistance mechanism ofl barteria can occur in a couple of ways.

1. Some bacteria share plasmids -- small chunks of DNA, like mini-chromosomes -- that exist outside the main chromosomes. This sharing can leap broad divisions in bacterial phylogeny . It's almost as if a cow could lend a crow a gene and teach it to grow teeth.

2. Gene cassettes are genes that can be spliced in the chromosomes. While the mechanism is kind of complex, it can be compared to an expedition to a shopping mall, Davies says. Genes called integrons code for enzymes called integrases that can splice those cassettes into chromosomes or other genetic material where they become functional.

In order to survive, bacteria develop sophisticated defence mechanisms to overcome the damaging effects of antibiotics (1-4) on their metabolism. Some bacteria have an innate natural resistance against particular antibiotics. Other bacteria acquire resistance after exposure to an antibiotic. In any event, resistance is one of the key concerns regarding the use of antibiotics. Acquired resistance may result from a mutation (genetic changes) in the bacterial chromosome, which will be passed on to the next generation(39), (42). More commonly, genetic material is transferred either directly or by a bacterial virus from another bacterium or even another bacterial species. Genetic material can be transferred as extra-chromosomal DNA (plasmid) or only as chromosomal fragments (transposons) (43). These genetic modifications result in four principles of the bacteria to cope with the 'deleterious' effect of the antibiotics. (a) They may alter the target site to prevent binding of the antibiotic to the site of action, e.g. the penicillin binding protein or gyrase, which is the major resistance mechanism for the fluoroquinolones. (b) Bacteria are able to produce enzymes, such as penicillinases and cephalosporinases, which inactivate beta-lactam antibiotics. Resistance against aminoglycosides is triggered by enzymes, which modify the antibiotic by acetylation, phosphorylation or adenylylation. Macrolide and glycopeptide resistance are due as well to the principle of target modification. Changes in the outer membrane permeability (porin) prevent antibiotics from entering the cell. This mechanism normally affects various antibiotic classes at the same time. (c) Bacteria can be capable to eliminate antibiotics rapidly from the cell by efflux pumps. Finally, sulfonamide and trimethoprim resistance can be achieved by bypassing the critical steps in the folic acid pathway. Bacteria are even capable, to develop various resistance mechanisms against an antibiotic at the same time. Once they have developed resistance, bacteria will survive exposure to the antibiotic. Usually bacteria concurrently acquire at least partial resistance (reduced sensitivity) to other antibiotics with similar structures and modes of action (1-4). Resistant strains multiply and then spread. These bacteria may be present only transiently or persist for long periods, sometimes causing major health problems. With the increasing prevalence of antimicrobial resistance among pathogens worldwide, resistance trends for all antibiotics have to be carefully and continuously monitored in order to safeguard the continued use of antibiotics(42).

The methods to be demonstrated antimicrobial susceptibility test (44).

(1) Agar diffusion with disk (Oxoid)

(2) Agar diffusion with E-test

(3) MIC-determination using commercially prepared microtitre trays with dehydrated antibiotics in wells (the Sensititre-system from Trek Diagnostic Systems) and using agar plates with 2-fold dilutions.

The MIC (Minimal Inhibitory Concentration) of a bacterium to a certain antimicrobial agent can be determined and gives the today best quantitative estimate for susceptibility. MIC is defined as the lowest concentration of antimicrobial agent required to inhibit growth of the bacteria. The principle is simple: Agar plates, tubes or microtitre trays with two-fold dilutions of antibiotics are inoculated with the bacteria and incubated. Next day the MIC is recorded as the lowest concentration of antimicrobial agent with no visible growth. The MIC informs you about the degree of resistance and might give you important information of the resistance mechanism and the resistance genes involved. MIC-determination performed as agar dilution is regarded as the golden standard for susceptibility testing. In contrast, diffusion tests are primarily qualitative methods that normally should only be used to report whether a bacterium is resistant or not. Principle: After an agar plate is inoculated with the bacteria, a tablet, disk or paperstrip with antimicrobial agent is placed on the surface. During incubation the antimicrobial agent diffuses into the agar and inhibits growth of the bacteria if sensitive. Diffusion tests are cheap compared to most MIC-determination methods. E-test is a diffusion test, but has been developed to give an approximately MIC-value. Well standardised methods are essential for all kinds of susceptibility testing, since the methods are highly sensitive to variations in several factors as for example size of inoculum, contents and acidity of the growth medium, time and temperature of incubation. The agar diffusion methods are also strongly influenced by factors as agar depth, diffusion rate of the antimicrobial agent and growth rate of the specific bacteria. The MIC-determination and disk diffusion methods described in this protocol are in accordance with the international recommendations given by the National Committee for Clinical Laboratory Standards (NCCLS) (45). The NCCLS describes how to perform the testing and set international guidelines for interpretation of the results. Quality control is regularly performed by running specific control strains as recommended by NCCLS (45). Some organisms are intrinsically

resistant to many (or even all) antimicrobial agents. Some microbiologists call this "primary resistance". Other organisms *acquire* resistance, either by mutating or by sharing the resistance genes of resistant organisms. This should not be confused with the term "clinical resistance" which refers to the failure of an antimicrobial to eradicate infection, despite the apparent ability of the agent to kill the 'bug' in vitro. A wide variety of problems can account for clinical resistance, such as impaired host immunity, inadequate drug delivery, and foreign material in the site of a wound(42).

Pulsed-field gel electrophoresis for separation of large DNA

Pulse field gel electrophoresis (PFGE) is a rather new technique that extends the molecular weight separation range so that large DNA molecules can be separated with very high resolution. The basic principle of Pulse field gel electrophoresis is the application of two electrical fields alternatively, at different angles for defined time periods. Separation is achieved by compelling large DNA molecules to continuously change direction as they proceed through the gel matrix. The advantages of this technique are an increase in the efficiency of the separation of the vital integrity of DNA molecules. The whole cells could be embedded in agarose gel(insert) which provide a natural and safe environment. The chromosomal DNAs are obtained in the inserts by subsequent digestion of all chromosomal proteins and other cell components. The inserts can be directly loaded into the well of the agarose gel to be run. Pulse field gel electrophoresis was specially designed to separate large DNA fragments. Consequently, the great innovation of PFGE analysis for mutation identification and diagnosis was its range of detection and its ability to scan for genetic rearragements at large distances from a given locus (probe) (46). For a specific disease, when closely linked genetic markers become available, PFGE analysis is used to determine the physical map of the region involved. Simultaneous analysis of patient material will quickly reveal if large chromosomal aberrations play a role in the development of the disease. Pulsed field gel electrophoresis (PFGE) has been used frequently for *Salmonella* genotyping and is the standard method used at the United States Centers for Disease Control and Prevention (21, 46-48). Several authors have specifically recommended using PFGE for *Salmonella* genotyping.

Determining which *Salmonella* isolates are derived from a common source is important in the identification of pathways of transmission between reservoirs. Isolates of *Salmonella* that are genetically similar share a recent common ancestor (i.e., common source), and the reservoirs from which they were sampled can be reasonably inferred to be either a source or destination for transmission. Therefore, the genetic similarity between isolates needs to be estimated accurately. Several methods are available for typing *Salmonella* isolates according to their genetic characteristics.

Tools for studying whole genomes

Conventional techniques for analysing DNA are designed for the analysis of small regions of whole genomes such as individual genes or operons. Many of the techniques used to study whole genomes are conventional molecular biology techniques adapted to operate effectively with DNA in a much larger size range (47).

1. PFGE

Agarose gel electrophoresis is a fundamental technique in molecular biology but is generally unable to resolve fragments greater than 20 kilobases in size – this makes it unsuitable for analysing DNA at a whole genome scale (whole microbial genomes are usually greater than 1000 kilobases in size). PFGE (pulsed field gel electrophoresis) is a adaptation of conventional agarose gel electrophoresis that allows extremely large DNA fragments to be resolved (up to megabase size fragments). PFGE is an essential technique for accurately determining the sizes of whole genomes/chromosomes prior to sequencing and is necessary for preparing large DNA fragments for large insert DNA cloning and analysis of subsequent clones. PFGE is also a commonly used and extremely powerful tool for genotyping and epidemiology studies for pathogenic microorganisms (49).

1.1 Principle of PFGE

Two factors influence DNA migration rates through conventional gels

1. Charge differences between DNA fragments

2. Molecular sieve effect of DNA pores

DNA fragments normally travel through agarose pores as spherical coils, fragments greater than 20 kb in size form extended coils and therefore are not subjected to the molecular sieve effect. The charge effect is countered by the proportionally increased friction applied to the molecules and therefore fragments greater than 20 kb do not resolve

- PFGE works by periodically altering the electric field orientation. The large extended coil DNA fragments are forced to change orientation and size dependent separation is re-established because the time taken for the DNA to reorient is size dependent
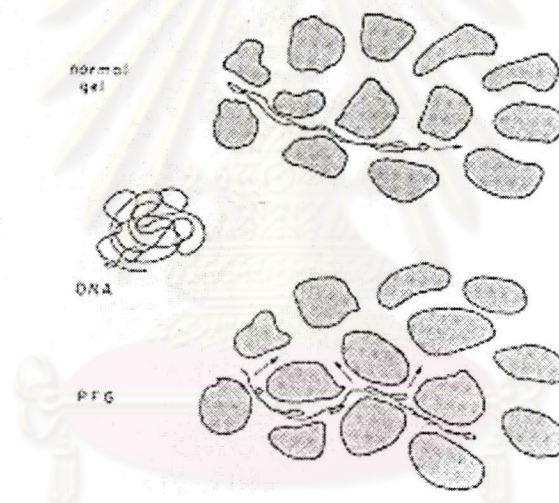
The most important factor in PFGE resolution is switching time, longer switching times generally lead to increased size of DNA fragments which can be resolved. Switching times are optimised for the expected size of the DNA being run on the PFGE gel. Switch time ramping increases the region of the gel in which DNA separation is linear with respect to size. A number of different apparatus have been developed in order to generate this switching in electric fields however most commonly used in modern laboratories are FIGE (Field Inversion Gel Electrophoresis) and CHEF (Contour-Clamped Homogenous Electrophoresis).

1.2 Preparation of DNA for PFGE

Ideally a genomic DNA preparation that contains a high proportion of completely or almost completely intact genome copies would be suitable for PFGE. Conventional means of DNA preparation are unsuitable for PFGE as mechanical shearing and low-level nuclease activity will result in fragmented DNA with an average size much smaller than an entire microbial genome (usually less than 200 kb in size). The solution to this is to prepare genomic DNA from whole cells in a semisolid matrix (ie. agarose) that eliminates mechanical shearing. A very high concentration of EDTA is also used at all times in order to eliminate all nuclease activity. Procedure of DNA preparation:

1) Intact cells are mixed with molten LMT agarose and set in a mold forming agarose 'plugs'

2) Enzymes and detergents diffuse into the plugs and lyse cells

3) Proteinase K diffuses into plugs and digests proteins

4) If necessary restriction digests are performed in plugs (extensive washing or PMSF treatment is required to remove proteinase K activity)

5) Plugs are loaded directly onto PFGE and run

For restriction digests, conventional enzymes are unsuitable as they cut frequently on an entire genome sequence producing DNA fragments that are far too small. Rare cutter restriction endonucleases cut genomic DNA with far less frequency than conventional restriction enzymes such as HindIII, BamHI etc. Many rare cutter RE's have 6-bp (or longer) recognition sites eg. NotI GC⇓GGCCGC. In many cases the frequency of cutting is highly species dependent eg. BamHI will cut far less frequently on a low GC% genome when compared to a intermediate or high GC content genome. Suitable rare cutter enzymes therefore have to be determined experimentally for each new species being studied (50).

2. Large insert cloning vectors – BAC's and PAC's

DNA cloning is another technique fundamental to molecular biology that requires adaptation in order to be useful in studying DNA at a whole genome scale. Conventional plasmid derived cloning vectors are only able to reliably maintain inserts less than 20 kb in size. There are a number of approaches to generating clones with inserts in an intermediate size range (20 – 80 kB) such as cosmids, etc. The most commonly used vectors for cloning extremely large DNA inserts are BAC's (Bacterial Artificial Chromosomes) and PAC's (P1-derived Artificial Chromosomes). Both BAC and PAC vectors are plasmid derived vectors distinguished from conventional vectors by extremely tightly controlled low copy numbers. These very low copy numbers help to limit the strain on host cellular resources generated by very large DNA inserts thus eliminating the rejection of large insert clones (50).