CHAPTER III

LITERATURE REVIEWS

I. Historical reviewed

The genus *Salmonella* is a group of bacterial pathogen in the family *Enterobacteriaceae* which original was called paratyphoid bacteria. The name '*Salmonella*" was assigned later to be honoring Salmon D. E. and Theobald Smith, american bacteriologists who were able to isolated *Salmonella* Choleraesuis from pig in 1884. In 1888, Gartner isolated *Salmonella* Enteritidis from spleen of human patient who sicked with endemic of foodborne disease in Germany. In 1892, Loffler isolated *Salmonella* Typhimurium from white mouse. The initial serodiagnostic of *Salmonella* was performed in 1986 by using the serum of salmonellosis human patient to agglutinate with the pathogen of salmonellosis human patient. Schottmiiller could distinguishing between *Salmonella* Paratyphi A and *Salmonella* Paratyphi B in 1900. According to increasing discovery of new strains of *Salmonella* from human patient and illness animal and developed of knowleage continue, 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 nomenclature and references of *Salmonella* serotyping is performed according to the Kauffman–White Scheme since 1955 (Edward, 1972) .

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* and updating the nomenclature of *Salmonella* serovar (Popoff *et al.*, 2001).

II. Bacterial characteristics

Salmonella species is a gram-negative rod shaped bacterial organism, ranging 0.7 to 1.5 x 2 to 5 μm in size. It belongs to a group of bacteria classified in the large family *Enterobacteriaceae*, tribe *Salmonelleae*. *Salmonella* species are motile by means of peritrichous flagella. They are facultatively anaerobic. Colonies are most often opaque and convex. *Salmonella* species are oxidase negative, catalase positive, indole and Voges Proskauer (VP) negative, methyl red and Simmon's citrate positive, H_2S producing and urease negative, non-lactose fermenting organisms (NLFs), D-glucose is fermented with the production of acid and usually gas. 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 table 1 (Ewing, 1986).

Salmonella species are also characterized by three major antigens by Kauffman–White (Popoff *et al.*, 1992)

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. O antigen of *Salmonella* is classified as Kauffman–White Scheme that each group of O antigen will be name in Arabic start from group A which composed of O:1, O:2, O:12 antigen, and respectively in other group until Z which is O:50. After Z group is O:51 to O:67 group respectively;

2. H antigen or flagella antigen, 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. The first phase will be name by small type letter start from a to z respectively, but nowadays found

Salmonella antigens

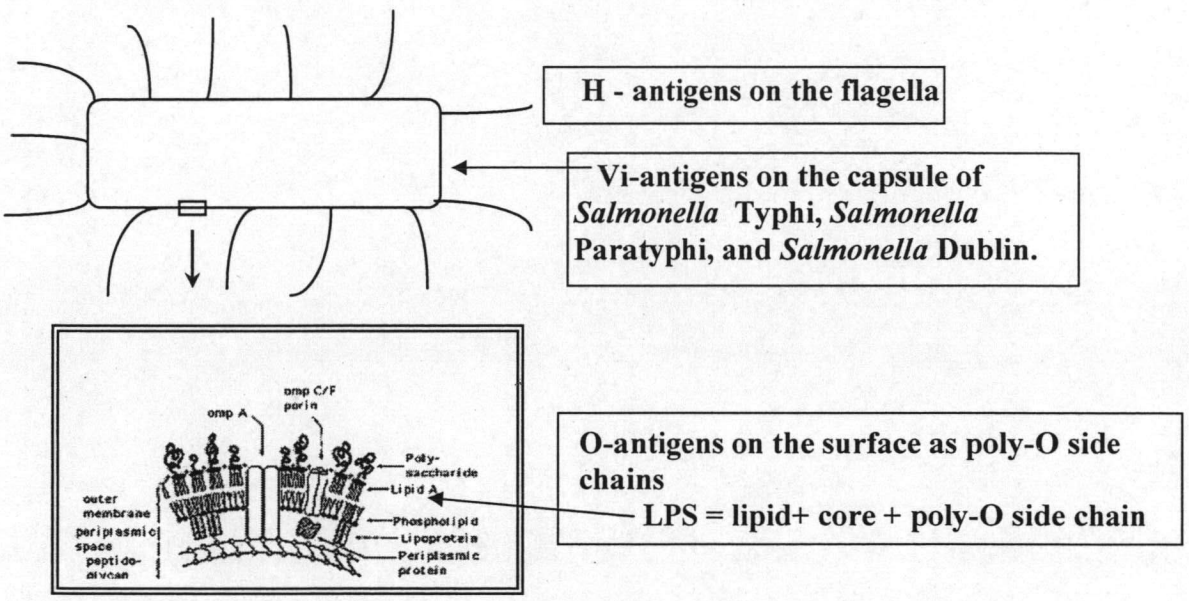

Figure1. Schematic drawing of *Salmonella* antigens.

many H antigen then must be name by z_1, z_2, ..., z_{68} and others. In case of second phase, there are many types and some types are difficult to detect. The important groups of single phase H–antigen strains such as *Salmonella* Paratyphi, *Salmonella* Typhi, *Salmonell*a Derby, ***Salmonella* Enteritidis**, *Salmonell*a Dublin and non-H-antigen such as *Salmonella* Gallinarum; and

3. Vi antigen is a special somatic antigen and belongs to the group of K antigens. *Salmonella* strains with Vi antigen have an greater pathogenicity than the strains without Vi antigen. The important strains with Vi antigen are *Salmonella* Typhi, *Salmonella* Paratyphi and *Salmonella* Dublin.

III. Taxonomy of the genus *Salmonella*, changes in serovars nomenclature

The genus *Salmonella* consists of two species: 1. *S. enterica* which is divided into six subspecies: *S. enterica* subspecies *enterica*, *S. enterica* subspecies *salamae, S. enterica* subspecies *arizonae, S. enterica* subspecies *diarizona, S. enterica* subspecies *houtenae,* and *S. enterica* subspecies *indica*; and 2. *S. bongori*. This nomenclature reflects the recent advances in *Salmonella* taxonomy (Le Minor, and Popoff, 1987). These species and subspecies can be distinguished on the basis of differential characters (Table 2) (Le Minor *et al.*, 1986).

Historically, species names were arbitrarily given to serovars for convenient reasons in medical practice. Some serovar names denoted syndrome (*S. typhi*) or relationship (*S. paratyphi* A, B, C). Other names were correlated with syndrome and host specificity which was right in some cases (*S. abortus-ovis, S. abortus-equi*) or wrong in other cases (*S. typhi-murium, S. cholerae-suis*). To avoid possible sources of confusion, names indicating geographical origin of the first strain of the new serovar (*S. london, S. panama, S. tel-el-kebir*) were then used. At the International Congress of Microbiology held in Moscow, it was decided that copound names would be hereafter condensed in

simple names (*S. typhimurium, S. choleraesuis, S. telelkebir*). These names, wrongly considered as species names, were for this reason italicized. They are in fact without taxonomic status, used to name bacteria frequently isolated in human or veterinary medicine. In other bacterial species (*Escherichia coli,* for example) names have not been given to serovars which are only designated by their antigenic formula. However, names of the most frequently encountered *Salmonella* serovars are so familiar that it would be unrealistic to suppress these names and to substitute their antigenic formula. Names were maintained only for subspecies *enterica* serovars which account for more than 99.5% of isolated *Salmonella* strains. These names must no longer be italicized. The first letter is a capital letter. In clinical practice the subspecies name does not need to be indicated as only serovars of subspecies *enterica* bear a name: Typhimurium, London or Montevideo are serovars of subspecies *enterica.* The name *Salmonella* serovar Typhimurium or *Salmonella* Typhimurium may be used for routine practice. Serovars of other subspecies of *S. enterica* and those of *S. bongori* are designated only by their antigenic formula.

Designation of the O groups.

The first-described O group were designated by letters of the alphabet. Later it was necessary to continue with numbers 51 to 67. It is now more logical to call each O group using the characteristic O factor. Letters are provisionally kept into brackets. For example, O:4 (B); O:18 (K). It is advisable to abandon the use of letters which are unnecessary for designating the first O groups, as listed in table 3.

Presentation of the scheme. Symbols

The serovars of *S. enterica* subsp. *enterica* are the name and for other subspecies of *S. enterica*, the subspecies to which belongs the serovar is indicated by the following symbol: and for the serovars of *S. bongori* was retained to avoid confusion with serovar name of *S. enterica* subsp. *enterica.*

Table 1. Differential biochemical reaction of *Salmonella*, *Citrobacter*, *E. tarda* and *Proteus*.

	Salmonella	*Citrobacter*	*E. tarda*	*Proteus*
TSI agar	K/A gas+, H_2S+	K/A gas+, $H_2S\pm$	K/A gas+, H_2S+	K/A,gas+,H_2S+
LDC	+	–	+	–
LDA	–	–	–	+
Urease	–	–	–	+
Mannitol	+	+	–	–

Table 2. Differential characters of *Salmonella species* and subspecies.

Species	***S. enterica***						***S. bongori***
Subspecies	*enterica*	*salamae*	*arizona e*	*diarizonae*	*houtenae*	*indica*	
Characters							
Ducitol	+	+	-	-	-	d	+
ONPG (2 h)	-	-	+	+	-	d	+
Malonate	-	+	+	+	-	-	-
Gelatinase	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	+	-	+
Culture with KCN	-	-	-	-	+	-	+
L(+)-tartrate[(a)]	+	-	-	-	-	-	-
Galacturonate	-	+	-	+	+	+	+
Gamma-glutamyl-transferase	+[(*)]	+	-	+	+	+	+
Beta-glucuronidase	d	d	-	+	-	d	-
	+	+	+	- (70%)	-	+	+
Mucate	-	-	-	-	+	-	-
Salicine	-	-	- (75%)	+ (75%)	-	d	-
Lactose	+	+	-	+	-	+	d
Lyse by phage O1							
Usual habitat	warm-blooded animals		Cold-blooded animals and environment				

(a) = *d*-tartrate

(*) = Typhimurium d, Dublin -.

\+ = 90% or more positive reactions

\- = 90% or more negative reactions

d = different reactions given by different serovars.

Table 3. Arrangement of *Salmonella* grouping must depend on O antigen.

Alphabetical	Present	Alphabetical	Present
A	2	L	21
B	4	M	28
C_1-C_4	6, 7	N	30
C_2-C_3	8	O	35
D_1	9	P	38
D_2	9, 46	Q	39
D_3	9, 46, 27	R	40
E_1-E_2-E_3	3, 10	S	41
E_4	1, 3, 19	T	42
F	11	U	43
G_1-G_2	13	V	44
H	6, 14	W	45
I	16	X	47
J	17	Y	48
K	18	Z	50

The symbols for somatic factors determined by phage conversion are underlined (example 6, 14, 18). They are present only if the culture is lysogenized by the corresponding converting phage. These factors are usually added to the factors present in non-converted strain (for example 6, 7 → 6, 7, 14). In O:3, 10 group, factors 15 or 15, 34 take the place of factor 10. For this reason, these factors are underlined and quoted into square brackets in this group. These underlined factors are mentioned in the table for serovars in which they were found. It is probable that this situation may be encountered for all serovars in the same group O.

[ ], O (not underlined) or H factor that may be present or absent without relation to phage conversion. Example: factor [5] of O:4 (B) group. When H factors are in square brackets, this means that they are exceptionally found in wild strains. For example, most strains of Paratyphi A possess a monophasic antigen phase 1 (a). In rare cases, diphasic strains with H: 1, 5 as H antigen phase 2 may be isolated. For this reason, [1, 5] is mentioned in square brackets in the formula of this serovar.

( ), O or H factor weakly agglutinable. The factor (k) corresponding to Arizona factor H:22 is weakly agglutinable by k standard serum, but is normally agglutinable by k polyvalent serum.

Presence or absence of accessory O factors (underlined or in square brackets) does not interfere with the serovar diagnosis. On the contrary, these factors are interesting as epidemiological markers for strains belonging to a some serovar.

The "R phases" of H antigens are uncommon. Their identification is usually done only by Reference Centers. Example for antigenic schema for *Salmonella* formulas which contain O antigen and H antigen are listed in table 4.

Information and references concerning the first isolation of each serovar.

They are collected in two books of Eckeart KELTERBORN: 1. *Salmonella*-species. S. Hirzel edit. Leipzig. German Democratic Republic, 1967.; and 2. catalogue of

Salmonella first isolation 1965-1984/Gustav Fisher edit. Iena. German Democratic Republic, 1987.

IV. Diseases caused by *Salmonella* and clinical manifestations

Some infectious disease texts recognize three clinical forms of salmonellosis: gastroenteritis, septicemia, and enteric fevers. The septicemic form of *Salmonella* infection can be an intermediate stage of infection in which the patient is not experiencing intestinal symptoms and the bacteria cannot be isolated from fecal specimens. The severity of the infection and whether it remains localized in the intestine or disseminates to the bloodstream may depend on the resistance of the patient and the virulence of the *Salmonella* isolate. The incubation period for *Salmonella* gastroenteritis (food poisoning) depends on the dose of bacteria. Symptoms usually begin 6 to 48 hours after ingestion of contaminated food or water and usually take the form of nausea, vomiting, diarrhea, and abdominal pain. Myalgia and headache are common; however, the cardinal manifestation is diarrhea. Fever (38°C to 39°C) and chills are also common. At least two-thirds of patients complain of abdominal cramps. The duration of fever and diarrhea varies, but is usually 2 to 7 days. Enteric fevers are severe systemic forms of salmonellosis. The best studied enteric fever is typhoid fever, the form caused by *Salmonella* Typhi, but any species of *Salmonella* may cause this type of disease. The symptoms begin after an incubation period of 10 to 14 days. Enteric fevers may be preceded by gastroenteritis, which usually resolves before the onset of systemic disease. The symptoms of enteric fevers are nonspecific and include fever, anorexia, headache, myalgias, and constipation. Enteric fevers are severe infections and may be fatal if antibiotics are not promptly administered (Black *et al.*, 1960, Rubin and Weinstein, 1977).

Table 4. 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. Choleraesuis	6,7	c	1,5
	S. Livingstone	6,7,14	d	l,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	-	-
	***S.* Enteritidis**	**1,9,12**	**g,m**	-
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
	S. Worthington	1,13,23	z	l,w
O: 38 (P)	*S.*Bangkok	38	Z_4z_{24}	-

Table 5. Presentation of the symbols's scheme and actual number of serovars in each species and subspecies.

Salmonella	**Symbol**	**Number of serovars**
S. enterica subsp. *enterica*	I	1,478
S. enterica subsp. *salamae*	II	498
S. enterica subsp. *arizonae*	IIIa	94
S. enterica subsp. *diarizonae*	IIIb	327
S. enterica subsp. *houtenae*	IV	71
S. enterica subsp.*indica*	VI	12
S. bongori	V	21
All total of serovars number of *Salmonella*		2,501

V. Pathogenesis

As with other gram-negative bacilli, the cell envelope of *Salmonella* contains a complex lipopolysaccharide (LPS) structure that is liberated on lysis of the cell and, to some extent, during culture. The lipopolysaccharide moiety may function as an endotoxin, and may be important in determining virulence of the organisms. This macromolecular endotoxin complex consists of three components, an outer O-polysaccharide coat, a middle portion (the R core), and an inner lipid A coat. Lipopolysaccharide structure is important for several reasons. First, the nature of the repeating sugar units in the outer O-polysaccharide chains is responsible for O antigen specificity; it may also help determine the virulence of the organism. *Salmonella* lacking the complete sequence of O-sugar repeat units are called rough because of the rough appearance of the colonies; they are usually a virulent or less virulent than the smooth strains which possess a full complement of O-sugar repeat units. Second, antibodies directed against the R core (common enterobacterial antigen) may protect against infection by a wide variety of gram-negative bacteria sharing a common core structure or may moderate their lethal effects. Third, the endotoxin component of the cell wall may play an important role in the pathogenesis of many clinical manifestations of gram-negative infections. Endotoxins evoke fever, activate the serum complement, kinin, and clotting systems, depress myocardial function, and alter lymphocyte function. Circulating endotoxin may be responsible in part for many of the manifestations of septic shock that can occur in systemic infections.

Salmonellosis includes several syndromes (gastroenteritis, enteric fevers, septicemia, fecal infections, and an asymptomatic carrier state) (Fig. 2). Particular serovars show a strong propensity to produce a particular syndrome (*Salmonella* Typhi, *Salmonella* Paratyphi-A, and *Salmonella* Schottmuelleri produce enteric fever; *Salmonella* Choleraesuis produces septicemia or focal infections; *Salmonella* Typhimurium and

Salmonella Enteritidis produce gastroenteritis); however, on occasion, any serotype can produce any of the syndromes. In general, more serious infections occur in infants, in adults over the age of 50, and in subjects with debilitating illnesses.

Most non-typhoidal *Salmonella* enter the body when contaminated food is ingested (Fig. 3). Person-to-person spread of *Salmonella* also occurs. To be fully pathogenic, *Salmonella* must possess a variety of attributes called virulence factors. These include, the ability to invade cells, a complete lipopolysaccharide coat, the ability to replicate intracellularly, and possibly the elaboration of toxin(s). After ingestion, the organisms colonize the ileum and colon, invade the intestinal epithelium, and proliferate within the epithelium and lymphoid follicles. The mechanism by which *Salmonella* invade the epithelium is partially understood and involves an initial binding to specific receptors on the epithelial cell surface followed by invasion. Invasion occurs by the organism inducing the enterocyte membrane to undergo "ruffling" and thereby to stimulate pinocytosis of the organisms (Fig. 4). Invasion is dependent on rearrangement of the cell cytoskeleton and probably involves increases in cellular inositol phosphate and calcium. Attachment and invasion are under distinct genetic control and involve multiple genes in both chromosomes and plasmids.

After invading the epithelium, the organisms multiply intracellularly and then spread to mesenteric lymph nodes and throughout the body via the systemic circulation; they are taken up by the reticuloendothelial cells. The reticuloendothelial system confines and controls spread of the organism. However, depending on the serotype and the effectiveness of the host defenses against that serotype, some organisms may infect the liver, spleen, gallbladder, bones, meninges, and other organs (Fig. 2). Fortunately, most serovars are killed promptly in extraintestinal sites, and the most common human *Salmonella* infection, gastroenteritis, remains confined to the intestine.

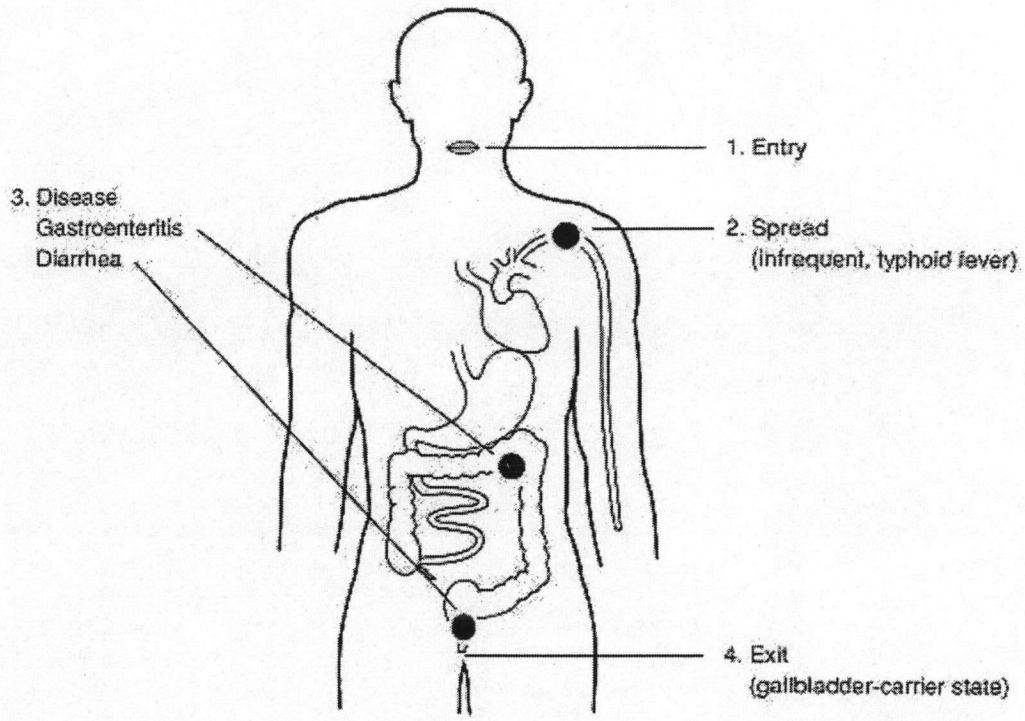

Figure 2. Pathogenesis of salmonellosis

After invading the intestine, most *Salmonella* induce an acute inflammatory response, which can cause ulceration. They may elaborate cytotoxins that inhibit protein synthesis. Whether these cytotoxins contribute to the inflammatory response or to ulceration is not known. However, invasion of the mucosa causes the epithelial cells to synthesize and release various proinflammatory cytokines, including: IL-1, IL-6, IL-8, TNF-2, IFN-U, MCP-1, and GM-CSF. These evoke an acute inflammatory response and may also be responsible for damage to the intestine. Because of the intestinal inflammatory reaction, symptoms of inflammation such as fever, chills, abdominal pain, leukocytosis, and diarrhea are common. The stools may contain polymorphonuclear leukocytes, blood, and mucus.

Much is now known about the mechanisms of *Salmonella* gastroenteritis and diarrhea. Figures 3 and 4 summarize the pathogenesis of *Salmonella* enterocolitis and diarrhea. Only strains that penetrate the intestinal mucosa are associated with the appearance of an acute inflammatory reaction and diarrhea (Fig. 5); the diarrhea is due to secretion of fluid and electrolytes by the small and large intestines. The mechanisms of secretion are unclear, but the secretion is not merely a manifestation of tissue destruction and ulceration. *Salmonella* penetrate the intestinal epithelial cells but, unlike *Shigella* and invasive *E. coli*, do not escape the phagosome. Thus, the extent of intercellular spread and ulceration of the epithelium is minimal. *Salmonella* escape from the basal side of epithelial cells into the lamina propria. Systemic spread of the organisms can occur, giving rise to enteric fever. Invasion of the intestinal mucosa is followed by activation of mucosal adenylate cyclase; the resultant increase in cyclic AMP induces secretion. The mechanism by which adenylate cyclase is stimulated is not understood; it may involve local production of prostaglandins or other components of the inflammatory reaction. In addition, *Salmonella* strains elaborate one or more enterotoxin-like substances which may stimulate intestinal secretion. However, the precise role of these toxins in the pathogenesis

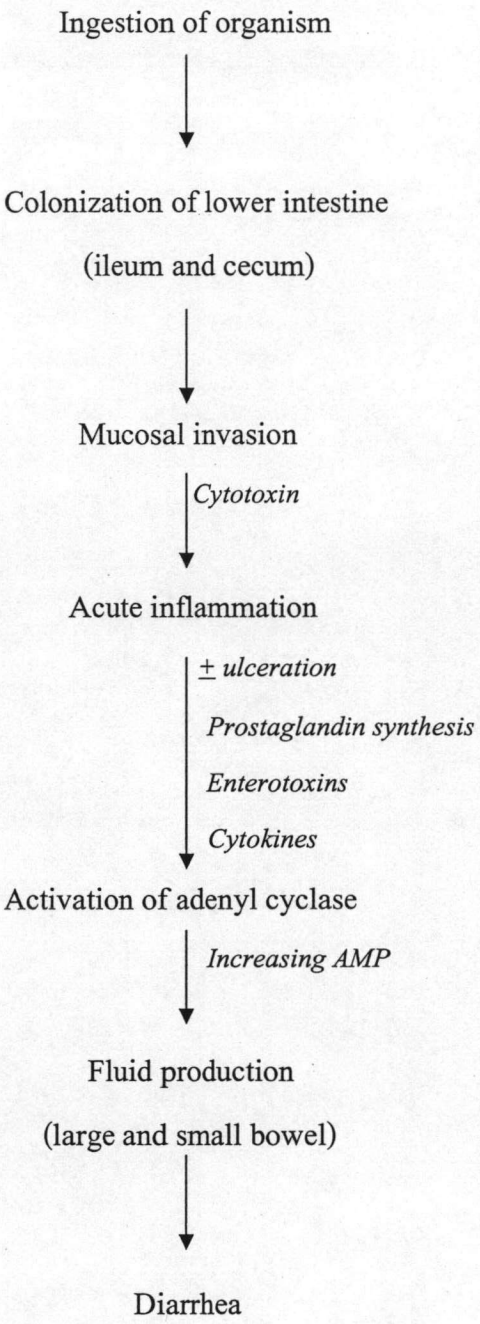

Figure 3. Scheme of the Pathogenesis of *Salmonella* enterocolitis and diarrhea.

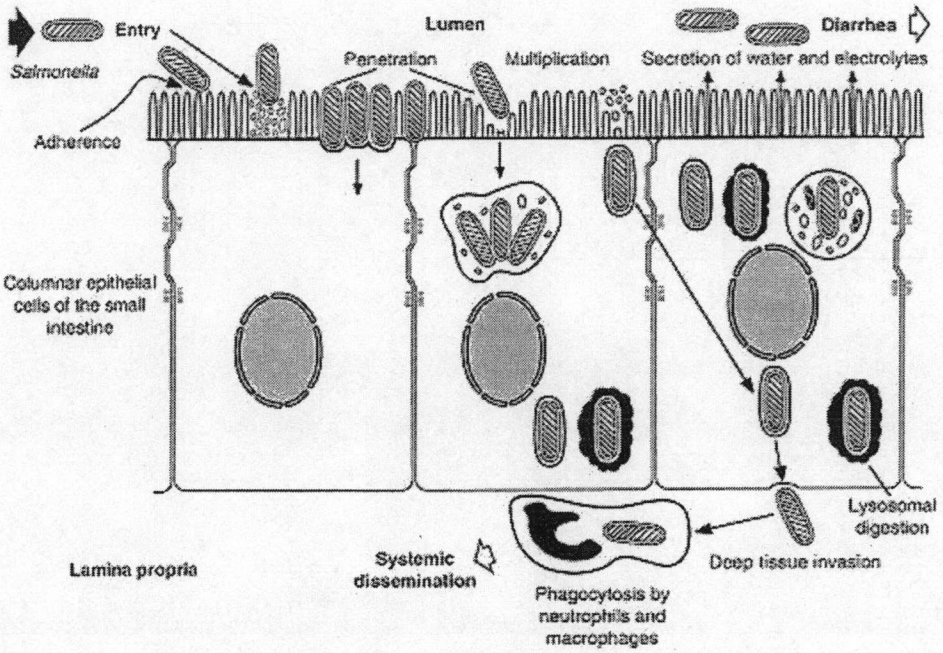

Figure 4. Invasion of intestinal mucosa by *Salmonella.*

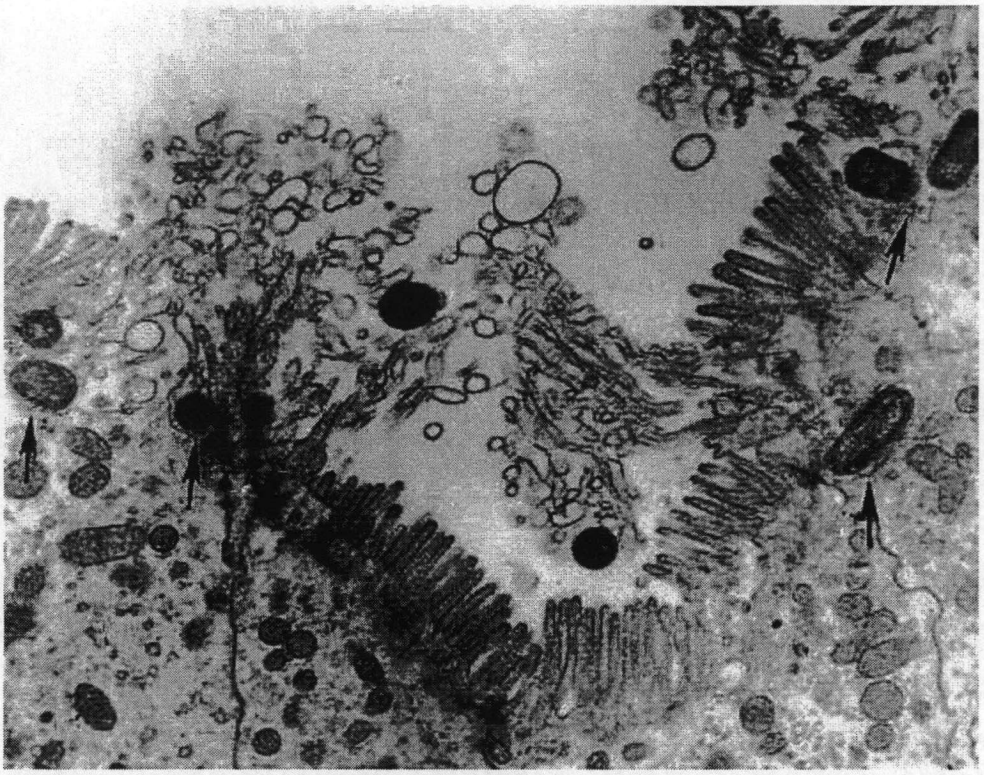

Figure 5. Electron photomicrograph demonstrating invasion of guinea pig ileal epithelial cells by *Salmonella* Typhimurium. Arrows point to invading *Salmonella* organisms. (Courtesy Akio Takeuchi, Walter Reed Army Institute of Research, Washington, D.C.).

of *Salmonella* enterocolitis and diarrhea has not been established (Chopra *et al.*, 1994; Finlay *et al.*, 1989; Finlay *et al.*, 1992; Galan and Curtiss, 1989; Giannella *et al.*, 1973; and Stephen *et al.*, 1985).

VI. Host Defenses

Various host defenses are important in resisting intestinal colonization and invasion by *Salmonella* (Table 6). Normal gastric acidity (pH < 3.5) is lethal to *Salmonella.* In healthy individuals, the number of ingested *Salmonella* is reduced in the stomach, so that fewer or no organisms enter the intestine. Normal small intestinal motility also protects the bowel by sweeping ingested *Salmonella* through quickly. The normal intestinal microflora protects against *Salmonella,* probably through anaerobes, which liberate short-chain fatty acids that are thought to be toxic to *Salmonella.* Alteration of the anaerobic intestinal flora by antibiotics renders the host more susceptible to salmonellosis. Secretory or mucosal antibodies also protect the intestine against salmonellae. Animal strains genetically resistant to intestinal invasion by salmonellae have been described. When these host defenses are absent or blunted, the host becomes more susceptible to salmonellosis; factors that render the host more susceptible to salmonellosis are listed in Table 7. For example, in AIDS, *Salmonella* infection is common, frequently persistent and bacteremic, and often resistant to even prolonged antibiotic treatment. Relapses are common. The role of host defenses in salmonellosis is extremely important, and much remains to be learned (Giannella *et al.*, 1975; Giannella, 1979).

Table 6. Host defenses against *Salmonella*.

Host defense	Examples of Factors
Gastric factors	Gastric acidity Rate of gastric emptying
Intestinal factors	Intestinal motility Normal intestinal flora Mucus Secretoty antibodies Genetic resistance to invasion
Nonspecific and other possible factors	Nutritonal state Lacioferrin Gut reticuloendothelial cells Lysozyme

Table 7. Factors increasing susceptibility to salmonellosis.

Location or factor	Specific condition
Stomatch	Achlorhydria Gastric surgery
Intestine	Antibiotic administration Gastrointestinal surgery ? Idiopathic imflammatory bowel disease
Hemolytic anemias	Especially sickle cell anemia and other hemoglobinopathies
Impaired systemic immunity	Caicinomatosis, leukemias, lymphomas Diabetes mellitus, Immunosuppressive drugs, acquired immunodeficiency syndrome (AIDS), etc

VII. Frequency

The US: *Salmonella* species cause estimated 1.4 million infection of each year or 560 cases per 100,000 inhabitants (Mead *et al.*, 2000).

Internationally: The Southeast Asia have reported the highest incidences of typhoid fever of the world which more than 1,000 cases per 100,000 inhabitants each year (Thong *et al.*, 1995).

Thailand: The annual estimation of human salmonellosis in Thailand is 76-1,043 cases per 100,000 inhabitants (Saithanoo and Bangtrakulnonth, 1998)

VIII. Epidemiology (Mishu *et al.*, 1994).

Contaminated food is the major mode of transmission for non-typhoidal *Salmonella* because salmonellosis is a zoonosis and has an enormous animal reservoir. The most common animal reservoirs are chickens, turkeys, pigs, and cows; dozens of other domestic and wild animals also harbor these organisms. Because of the ability of *Salmonella* to survive in meats and animal products that are not thoroughly cooked, animal products are the main vehicle of transmission. The magnitude of the problem is demonstrated by the following recent yields of *Salmonella*: 41% of turkeys examined in California, 50% of chickens cultured in Massachusetts, and 21% of commercial frozen egg whites examined in Spokane, WA.

The epidemiology of typhoid fever and other enteric fevers primarily involves person-to-person spread because these organisms lack a significant animal reservoir. Contamination with human feces is the major mode of spread, and the usual vehicle is contaminated water. Occasionally, contaminated food (usually handled by an individual who harbors *Salmonella* Typhi) may be the vehicle. Plasmid DNA fingerprinting and

bacteria phage lysotyping of *Salmonella* isolates are powerful epidemiologic tools for studying outbreaks of salmonellosis and tracing the spread of the organisms in the environment.

In typhoid fever and non-typhoidal salmonellosis, two other factors have epidemiologic significance. First, an asymptomatic human carrier state exists for the agents of either form of the disease. Approximately 3% of persons infected with *Salmonella* Typhi and 0.1% of those infected with non-typhoidal *Salmonella* become chronic carriers. The carrier state may last from many weeks to years. Thus, human as well as animal reservoirs exist. Interestingly, children rarely become chronic typhoid carriers. Second, use of antibiotics in animal feeds and indiscriminant use of antibiotics in humans increase antibiotic resistance in *Salmonella* by promoting transfer of R factors.

Salmonellosis is a major public health problem because of its large and varied animal reservoir, the existence of human and animal carrier states, and the lack of a concerted nationwide program to control *Salmonella.*

IX. Diagnosis

The diagnosis of salmonellosis requires bacteriologic isolation of the organisms from appropriate clinical specimens. Laboratory identification of the genus *Salmonella* is done by biochemical tests; the serologic type is confirmed by serologic testing. Feces, blood, or other specimens should be plated on several nonselective and selective agar media (blood, MacConkey, eosin-methylene blue, bismuth sulfite, *Salmonella-Shigella,* and brilliant green agars) as well as intoenrichment broth such as selenite or tetrathionate. Any growth in enrichment broth is subsequently subcultured onto the various agars. The biochemical reactions of suspicious colonies are then determined on triple sugar iron agar and lysine-iron agar, and a presumptive identification is made. Biochemical identification

of *Salmonella* has been simplified by systems that permit the rapid testing of 10-20 different biochemical parameters simultaneously. The presumptive biochemical identification of *Salmonella* then can be confirmed by antigenic analysis of O and H antigens using polyvalent and specific antisera. Fortunately, approximately 95% of all clinical isolates can be identified with the available group A-E typing antisera. *Salmonella* isolates then should be sent to a central or reference laboratory for more comprehensive serologic testing and confirmation.

X. Control

Salmonella are difficult to eradicate from the environment. However, because the major reservoir for human infection is poultry and livestock, reducing the number of *Salmonella* harbored in these animals would significantly reduce human exposure. In Denmark, for example, all animal feeds are treated to kill *Salmonella* before distribution, resulting in a marked reduction in salmonellosis. Other helpful measures include changing animal slaughtering practices to reduce cross-contamination of animal carcasses; protecting processed foods from contamination; providing training in hygienic practices for all food-handling personnel in slaughterhouses, food processing plants, and restaurants; cooking and refrigerating foods adequately in food processing plants, restaurants, and homes; and expanding of governmental enteric disease surveillance programs.

The U.S. Department of Agriculture has approved the radiation of poultry to reduce contamination by pathogenic bacteria, e.g. *Salmonella* and *Campylobacter.* Unfortunately, radiation pasteurization has not yet been widely accepted in the U.S. Adoption and implementation of this technology would greatly reduce the magnitude of the *Salmonella* problem.

Vaccines are available for typhoid fever and are partially effective, especially in children. No vaccines are available for non-typhoidal salmonellosis. Continued research in this area and increased understanding of the mechanisms of immunity to enteric infections are of great importance.

General salmonellosis treatment measures include replacing fluid loss by oral and intravenous routes, and controlling pain, nausea, and vomiting. Specific therapy consists of antibiotic administration. Typhoid fever and enteric fevers should be treated with antibiotics. Antibiotic therapy of non-typhoidal salmonellosis should be reserved for the septicemic, enteric fever, and focal infection syndromes. Antibiotics are not recommended for uncomplicated *Salmonella* gastroenteritis because they do not shorten the illness and they significantly prolong the fecal excretion of the organisms and increase the number of antibiotic-resistant strains.

XI. When to use strain typing

Bacterial strain typing data are most effective when they are collected, analyzed, and integrated into the results of an epidemiological investigation. The epidemiologist should initiate strain typing studies in consulation with the infection control laboratory or the microbiology staff when investigating a potential outbreak of an infectious disease (McGowan and Metchock, 1995). This may be triggered by a noticeable increase in the rate of isolation of a particular pathogen, the recognition in the clinical microbiology laboratory of multiple isolates with an unusual biotype or antibiogram. Strain typing data should supplement, and not replace, a carefully conducted epidemiological investigation. In some cases, typing data can effectively rule out and outbreak and thus avoid the need for an extensive epidemiological investigation. In other cases, strain typing data may reveal the presence of outbreaks caused by more than one

strain. However, undue reliance on strain typing in the absence of epidemiological data is an inefficient use of laboratory resources.

XII. Typing methods in the epidemiologic study

Typing methods fall into broad categories: phenotypic methods and genotypic methods. Phenotypic methods are those that characterize the products of gene expression in order to differentiate strains. Properties such as biochemical profiles, bacteriophage typing (phage typing), antigens present on the cell's surface, and antimicrobial susceptibility profiles. All are examples of phenotypic properties that can be determined in the laboratory. Because they involve gene expression, these properties all have a tendency to vary, based on changes in growth conditions, growth phase, and spontaneous mutation.

Genotypic methods are those that are based on an analysis of the genetic structure of an organism and include polymorphisms in DNA restriction patterns based on cleaveage of the chromosome by enzymes that cleave the DNA into 10 to 30 fragments (infrequent cutters), and the presence or absence of extrachromosomal DNA. Genotypic methods are less subject to natural variation, although they can be effected by insertions or deletions of DNA into the chromosome, the gain or loss of extrachromosomal DNA, or random mutations that may create or eliminate restriction endonuclease sites.

All typing systems can be characterized in terms of typeability, reproducibility, discriminatory power, and ease of interpretation. The characteristics of a number of typing methods are presented in table 8 (Arbeit, 1995). Typeability refers to the ability of a technique to assign an unambiguos result (type) to each isolate. Although nontypeable isolates are more common with phenotypic methods, they have been recognized with most methods. For example, with PFGE, a technique that is almost uniformly applicable to bacteria, some strains of *Clostridium difficile* remain nontypeable because the

Table 8. Characteristic of typing systems.

Typing systems	Proportion of strains typeable	Reproducibility	Discriminatory power	Ease of performance
Biotyping	All	Poor	Moderate	Easy
Antimicrobial susceptibility patterns	All	Good	Easy	Easy
Serotyping	Most	Good	Moderate	Moderate
Plasmid fingerprinting	Most	Good	Moderate	Moderate
REA of cDNA with conventional electrophoresis	All	Good	Difficult	Moderate
RFLP analysis with DNA probes	All	Excellent	Moderate	Difficult
PFGE	All	Excellent	Moderate	Moderate
AP-PCR	All	Good	Moderate	Moderate

Data from Arbeit, R.D. Manual of Clinical Microbiology, 1995.

Abbreviations: REA, restriction endonuclease analysis; cDNA, chromosomal DNA; RFLP, restriction fragment-length polymorphism; PFGE, pulsed-field gel electrophoresis; AP-PCR, arbitrarily primed polymerase chain reaction.

chromosomal DNA is degreaded, presumably by endogenous nuclease, before it can be cleaved properly by the restriction endonucleases used in the PFGE protocol.

A reproducible method is one that yields the same results upon repeat testing of a bacterial strain. In the context of an epidemiological study, this means that the same strain recovered from epidemiologically linked patients will give the identical (or nearly identical) typing result. Poor reproducibility may reflect technical variation in the method or biologic variation occurring during *in vivo* or *in vitro* passage of the organisms to be examined. Over time (a few weeks to years, depending on the species), the typing patterns produced by DNA-based methods, such as PFGE and AP-PCR, will show some minor, natural variation. Thus, when analyzing results, it is important to consider the length of time over which the bacterial isolates were collected.

The discriminatory power of a technique refers to its ability to differentiate among epidemiologically unrelated isolates, ideally assigning each to a different type. Traditional phenotypic methods, such as antibiogram typing, serotyping, and biotyping, frequently show lower discriminatory power than newer molecular methods.

Ease of performance reflects the cost of specialized reagents and equipment the technical complexity of a method, and the effort required to learn and to implement the technique in the laboratory. Most molecular methods required the purchase of new equipment, some of which is costly ($4,000-$20,000)(Tenover *et al.*, 1997). However, these methods are learned easily and are widely applicable to a variety of species. Many traditional methods also involve considerable costs in labor and materials, but are restricted to a single or relatively few species. For example, bacteriophage typing, which is used primarily for *Staphylococcus aureus* and a few other bacterial species, requires the maintenance of bacteriophage stocks that constantly must be replenisshed and titered, a process that is both time-consuming and labor-intensive.

Finally, ease of interpretation refers to the effort and experience required to obtain useful, reliable typing information using a particular method. At present, the interpretation of the results of molecular methods remains an area of active discussion. However, this is contrast to methods such as bacteriophage typing and pyocin typing, which require significant expertise to perform and interpret and often still yield ambiguous results.

1. Phenotyping methods

Typing methods that assess phenotypic differences are inherently limited by the capacity of microorganisms to alter the expression of the underlying genes. Such changes may occur unpredictably or in response to various environmental stimuli. In addition, point mutations representing a single nucleotide in the entire chromosome can result in the abnormal regulation or function of the gene responsible for a particular phenotype. Thus, isolates that represent the same strain and that are genetically indistinguishable (or almost so) can vary in the phenotype detected.

1.1. Biotyping

In the 1960s and early 1970s, identification of bacterial species was frequently undertaken using racks of tubes representing a variety of biochemical tests, and the variability of certain tests, such as indole, H_2S, or pigment production, served as markers for particular strains. Thus, biotyping emerged as a useful tool for the epidemiological investigations. At present, the identification of bacterial species normally is accomplished by using a combination of biochemical and immunologic tests of which now are performed using commercial kits or automated devices. However, biotyping using automated methods relies on a variety of novel substrates, and some of these tests, such as carbohydrate fermentations, are highly variable even within isolates of the same strain. Thus, biotyping, like most phenotyping methods, has only modest reproducibility, because

microorganism can alter unpredictably, the expression of many cellular products. Moreover, contempolary biotyping typically has poor discriminatory power and cannot differentiate among some of the current nosocomial problem pathogens, such as enterococci, where biochemical diversity is uncommon. Occasionally, outbreaks occurred when many cases of infections caused by bacterial strains that represent unusual species or unusual biotypes of common species, for example, H_2S-producing isolates of *Escherichia coli* were observed. In such situations, additional typing, techniques may not be needed. However, even cluster of unusual isolates may not always indicate a common-source outbreak, as indicated by a recent report (Tenover *et al*, 1997) in which four isolates of *Leptotrichia buccalis*, an unusual anaerobic gram-negative bacillus, recovered from blood cultures of four different bone marrow transplant patients, were found to be unrelated by PFGE and fatty-acid profile analysis. The investigation of the suspected outbreak revealed that each of the patients had undergone dental manipulations prior to developing bacteremia. In addition, all of the patients had been placed on prophylactic antimicrobial agents to which the *Leptotrichia buccalis* isolates were resistant. Thus, each neutropenic patient developed bacteremia with his own endogenous strain of *Leptotrichia buccalis*, which served as an opportunistic pathogen. Nonetheless, it should be noted that outbreaks can, in some cases, be caused by multiple pathogens.

1.2. Antimicrobial susceptibility patterns

Antimicrobial susceptibility 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 (e.g. transposons and plasmid). Changes in antibiograms also may reflect spontaneous point mutations, such as seen with fluoroquinolones. Thus, isolates that are epidemiologically related and otherwise genetically indistinguishable may manifest

different antimicrobial susceptibilities due to acquisition of new genetic material over time or the loss of plasmids. Conversely, unrelated isolates may have indistinguishable resistance profiles, which may represent acquisition of the same plasmid by multiple species (a "plasmid outbreak").

1.3. Serotyping

Serotyping, a nonmolecular method, uses a series of antibodies to detect different antigenic determinants on the surface of the bacterial cell. Serotyping is the one of the classic strain typing techniques that has been used over the years for the epidemiological studies of many species of bacteria. It remains a key method for typing isolates of *Salmonella, Shigella,* and pneumococci. However, maintaining stocks of typing sera (including the >2,500 antisera required for definitive *Salmonella* typing) is a major limitation of this method. Because of the association of certain *Salmonella* serotypes with foodborne disease, and the association between specific pneumococcal serotypes and invasive disease, particularly in children, serotyping continues to be a valuable typing technique. Nonetheless, PFGE has been shown to resolve distinct clonal strains within individual serotypes of both *Salmonella* and pneumococci, thus indicating that it is a more discriminatory typing tool.

1.4. Bacteriophage or Phage Typing.

It is one of the most discriminating phenotypic typing systems. Bacteriophage are viruses that infect bacteria. Susceptibility to lysis requires presence of phage receptor. A single phage can replicate 200 fold in 15-20 minutes and then lyses the host cell (releases phage particles). Plaques of growth inhibition can be seen on an indicator lawn of sensitive cells. Phage binding to an *Escherichia coli* cell. Phage plaques in bacteria lawn. Used in reference laboratories for typing some important pathogens such

as *Salmonella species, Shigella species* and *Staphylococcus aureus.* Susceptibility to lysis by a defined set of bacteriophages. Limitations of this method are incomplete typeability, poor reproducibility and genetic instability of phage typing.

1.5. Gel Electrophoretic methods

Several electrophoretic methods based on cellular and surface protein patterns have been developed to be the very useful investigative tools; these include polyacrylamide gel electrophoresis combined with the radiolabeling or immunoblotting.

(a) Radio-polyacrylamide gel electrophoresis or PAGE of (^{35} S) methionine-labeled proteins. This method is based on the incorporation of (^{35} S) methionine into cellular proteins, the separation of these proteins on SDS-PAGE, and subsequent autoradiography.

(b) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

These methods are cheap to perform. Limitations of the method are difficult to standardise and complex protein profiles are difficult to interpretation.

1.6. Multilocus Enzyme Electrophoresis (MLEE)

Multilocus enzyme electrophoresis is a technique that combines electrophoresis in a starch gel matrix with specific enzyme staining. With MLEE, variations in functional enzymes (allozymes) are detected in stained starch gels following electrophoresis. Positive activity is visually detected as bands in the gels. Presence or absence (null alleles) of activity is scored, as is the mobility of those bands present. Mobility differences among strains of bacteria are due to a difference in net charge of the enzyme, which is a result of the sum total of the amino acids that comprise the enzyme. After a number of enzymes are evaluated, a bacterial strain profile (electrophoretic type) is

realized.

2. Genotypic methods

Over the last several years, various molecular techniques have emerged as the methods of choice for typing bacterial isolates. They are plasmid fingerprinting; restriction endonuclease analysis (REA) of plasmid DNA; REA of chromosomal DNA using frequent cutting enzymes and conventional electrophoresis; restriction fragment-length polymorphism (RFLP typing) analysis using DNA probe; AP-PCR and other related nucleic acid amplification-based typing methods; and PFGE. The detail of each method was described as followed.

2.1. Plasmid fingerprinting

Plasmid fingerprinting was the first molecular method to be used as a bacterial typing tool. Plasmids are extrachromosomal DNA elements that are present in most clinical isolates and can be identified readily by simple cell lysis procedures followed by agarose gel electrophoresis of the lysates (Fig. 6)(Tenover, 1985). The number and size of the plasmid present is used as the basis for strain identification. This strain typing technique has been used successfully for analysis of the outbreaks of nosocomial infections and community-acquired infections caused by a variety of species of gram-negative rods.

2.2. REA of plasmid DNA

Some strains of bacteria contain only a single large plasmid, often in the size range of 100 to 150 kilobases (kb). Because it is difficult to differentiate plasmids in this size range, especially those that vary by only 10 kb to 15 kb, some investigators have added a restriction endonuclease digestion step to try to increase the discriminatory power

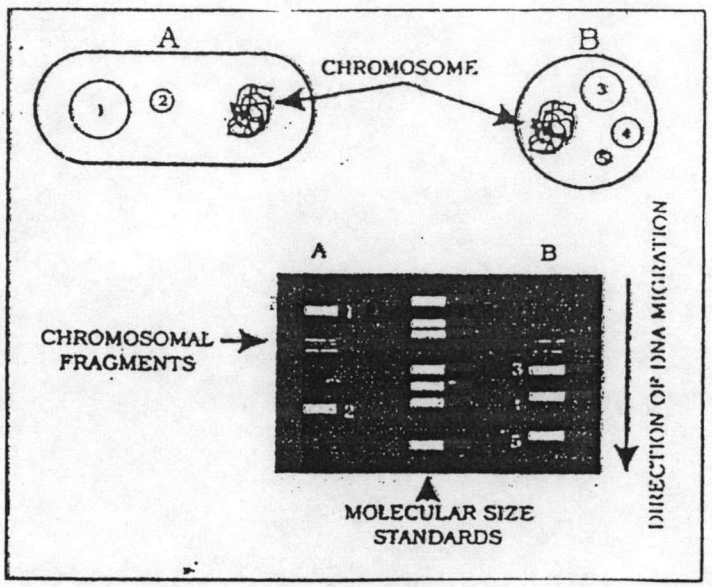

Figure 6. Schematic drawing of the plasmid fingerprinting technique using agarose gel electrophoresis. The oval on the left signifies a typical gram-negative rod, and the circle on the right signifies a typical gram-positive coocus. Cell are lysed using detergents at high pH, the chromosomal DNA is removed, and the plasmid DNA is applied to an agarose gel that is stained and photographed. Some chromosomal fragments usually are visible on the gel and serve as an internal molecular size standard (approximately 12-15). Plasmid DNA within the bacteria is shown in circular form. Each plasmid is numbered within the cell, and its corresponding position in the agarose gel is indicated by the same number (Tenover,1985).

of agarose gel electrophoresis (Fig. 7)(Pfaller *et al.*,1991). While this can be helpful, large plasmids produce many restriction fragments, which can make interpretation more difficult, espectially when the multiple large plasmids are present. Thus, for gram-negative rods, the REA step is no longer performed in most laboratories. However, for analysis of staphylococci, where the plasmids typically are <50 kb, REA appears to increase the discriminatory power of the analysis, because the number of restriction fragments generated usually is <20 fragments. Digestion also makes the patterns of the restriction fragments produced from staphylococcal plasmids easier to analyze than the undigested profiles, which often show multiple forms for plasmids of less than 15 kb, because circular and linear forms of the plasmids migrate at different rates than the covalently closed circular form. Plasmid fingerprinting is technically simple to perform and requires relatively inexpensive equipment ($1,500-$3,000)(Tenover *et al.*, 1997). At this time, the method is used primarily as an alternative technique for staphylococcal isolates, which frequently carry multiple plasmids, and for selected species of *Enterobacteriaceae,* which often have large distinctive plasmids.

When applying the plasmid fingerprinting technique, investigators must be aware of two confounding factors. First, it is possible that plasmids can spread to multiple species of bacteria, causing a plasmid outbreak in which unusual antibiograms are recognized in multiple species. This has been recognized both in gram-negative rods and in staphylococci. Second, it is important to appreciate that the structure of individual plasmids and the plasmid content of a particular strain may vary over time. This variability reflects two factors: over time, plasmids can be lost spontaneously or acquired from other organisms, and plasmids frequently carry smaller mobile genetic elements (transposon and insertion sequences) that promote duplications and deletions of DNA segments. Both plasmids and transposon often include antimicrobial resistance determinants and thus are subject to considerable selective pressure within hospitals due to antimicrobial agent use.

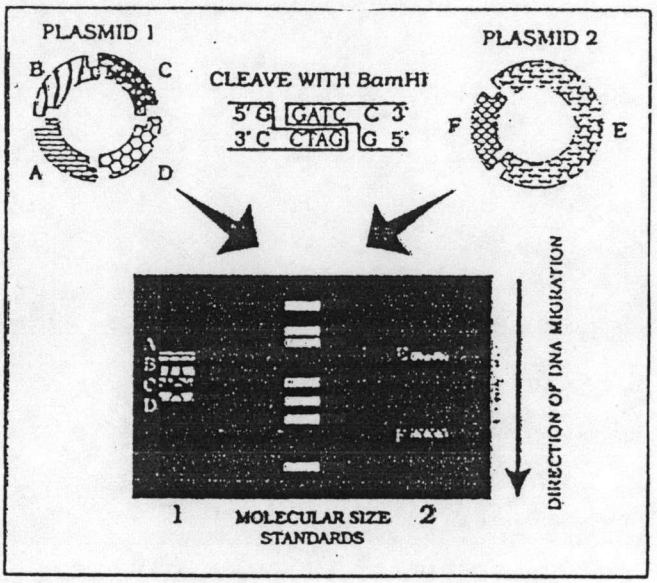

Figure 7. Schematic drawing of restriction endonuclease digestion of two unique plasmid, followed by agarose gel electrophoresis. Different sized restriction fragments are denoted by the different patterns (Pfaller *et al.*,1991).

In general, plasmid fingerprinting is most useful for epidemiological studies that are limited both temporally and geographically. In selected instances, plasmid fingerprinting may complement other techniques, such as PFGE analysis, by providing a basis for differentiating isolates that are related genotypically but are separated epidemiologically by moderate time periods, such as several months.

2.3. Gel electrophoresis techniques for analysis of chromosomal DNA

There are two methods of typing organisms based on fragment patterns produced by cleaving chromosomal DNA with restriction endonucleases. The first method, often referred to as conventional electrophoresis, uses a restriction enzyme that cuts the chromosome into hundreds of pieces (frequent cutter), followed by standard agarose gel electrophoresis. Fragments that are 25 kb to 0.5 kb are resolved into a discernible banding pattern, although a single band may contain fragments of similar size from several different areas of the chromosome. Larger fragments coalesce at the top the gel or do not migrate into the gel. The second method, PFGE, uses enzymes that cut chromosomal DNA infrequently, generating from 10 to 30 bands, followed by a novel from of electrophoresis that can separate fragments from 1 kb up to 1,000 kb (1 megabase). Each method, and a vaiation of the conventional electrophoresis method, is described in greater detail below.

2.3.1. REA of chromosomal DNA with frequent cutting enzymes and conventional electrophoresis

Each restriction endonuclease cleaves DNA at a particular sequence of nucleotides that may be repeated numerous times around the chromosome. The number and size of the restriction fragments generated by digesting a given piece of DNA reflects the frequency and distribution of the restriction sites. In conventional REA, endonucleases

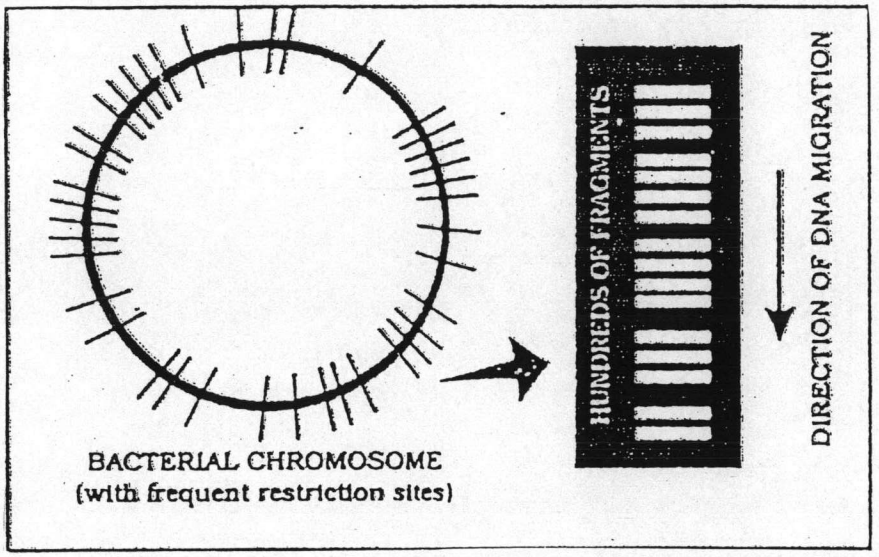

Figure 8. Schematic drawing of restriction endonuclease analysis of chromosomal DNA using conventional electrophoresis. The box on the right represents the banding pattern of hundreds of fragments after conventional agarose gel electrophoresis. Each band may contain a number of unique chromosomal fragments of similar size (Swaminathan and Mater,1993).

with frequently occurring sites in the bacterial genome are used to digest total DNA (plasmid and chromosome), thereby generating hundreds of fragments ranging from approximately 0.5 to 50 kb in length (Fig. 8)(Swaminathan and Mater, 1993). Such fragments can be separated by size using agarose gel electrophoresis, and the pattern can be detected by staining the gel with ethidium bromide (or other dyes) and photographing under ultraviolet light. Different strains of the same bacterial species have different REA profiles (depicted as a series of bands on agarose gels) because of variations in their DNA sequences. All isolates are typeable by REA; however, it can be very difficult to interpret the complex profiles, which consist of hundreds of bands that may be indistinct or overlapping. Although the approach has been applied to many species, at this time, its primary use is as an alternative technique for analyzing *Clostridium difficile.*

2.3.2. PFGE

Pulsed-field gel electrophoresis was first described in 1984 as a tool for examining the chromosomal DNA of eukaryotic organisms (Fig. 9) (Schwartz and Cantor, 1984). Subsequently, PFGE has proven to be a highly effective molecular typing technique for many different bacterial species.

Types of PFGE

PFGE size resolves DNA molecules of a millimeter in length through the use of pulsed-field, which selectively modulate mobilities in a size-dependent fashion. The pulsed electrophoresis effect has been utilized by a variety of instrument (FIGE, TAFE, CHEF, OFAGE, PACE, and rotating electrode gel) to increase the size resolution of both large and small DNA molecules (Soleyman Demirel University, 2001). It is important when choosing a PFGE system to evaluate cost and performance in the light of projected use. There are different types of PFGE. There are:

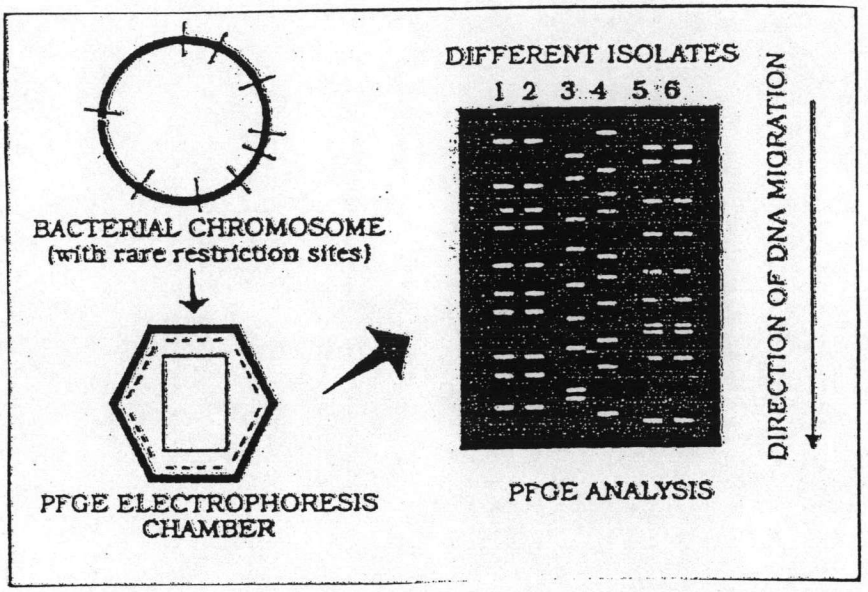

Figure 9. Schematic drawing of pulsed-field gel electrophoresis, in which chromosomal DNA is cleaved with a rare cutting enzyme followed by electrophoresis, using a unique chamber and current switching protocol. The box on the right is the agarose gel showing the very large DNA fragments derived from the unique electrophoresis chamber (Schwartz and Cantor, 1984).

Field-Invertion Gel Electrophoresis (FIGE): In 1986, Carle, Frank and Olsen developed a simpler system, FIGE, in which the two fields were 180° apart. Electrode polarity was reversed at intervals, with a longer forward than reverse pulse time to generate a net forward sample migration. Net forward migration is achieved by increasing the ratio of forward to reverse pulse times to 3:1. To improve the resolution of the bands by FIGE, the duration of pulse times is increased progressively during a run. This is called "switch time ramping". By changing pulse durations continually during the course of an experiment, FIGE has the advantages of straight lanes and simple equipment. All that is needed are standard gel boxes and a pulse controller. Today, FIGE is very popular for smaller fragment separations. FIGE provides acceptable resolutionup to 800 kilobases (600-750 kb).

Tranverse-Alternating Field Electrophoresis (TAFE): This form of PFGE allows separation of large DNA fragments in a sample, convenient format without the drawbacks of earlier pulsed-field techniques. In TAFE, the gel is oriented vertically and a simple four-electrode array is placed not in the plane of the gel, but in front and at the back of it. Sample molecules are forced to zigzag through the thickness of the gel, and all lanes experience the same effects so the bands remain straight. As the molecules moves down the gel, they are subjected to continual variations in field strength and reorientation angle, but to all lanes equally. However, the angle between the electric fields varies from the top of gel (115°) to the bottom (approximately 165°) and hence molecules still do not move at a constant velocity over the length of the gel. TAFE technology, with regular and sharp separation of DNA bands, will be of special advantage in the study of genetics of many pathogenic protozoans, where such analysis was impossible before. TAFE has been used for the separation of fragments up to 1,600 kilobase fragments.

Contour-Clamped Homogeneous Electric Fields (CHEF): CHEF is the most widely used apparatus. The CHEF apparatus provides a more sophisticated solution to the distorting effects of both the edges of the chamber and the passive electrodes. CHEF has twenty-four point electrodes equally spaced around the hexagonal contour. In the CHEF system, there are no "passive" electrodes. All the electrodes are connected to the power supply via an external loop of resistor, all of which have the same resistance. This loop is responsible for setting the voltages of all the electrodes around the hexagonal contour to values appropriate to the generation of uniform fields in each of the alternate switching position. The CHEF system sets the voltages at these 24 points. This apparatus produces electric fields that are sufficiently uniform so that all lanes of the gel run straight. CHEF uses an angle of reorientation of 120° with gradiations of electropotential radiating from the positive to negative pores. Molecules up to 7,000 kb can be separated by CHEF.

Orthogonal-Field Alternating Gel Electrophoresis (OFAGE): A similar apparatus that used two nonhomogeneous electric fields was reported by Carle and Olson in 1984. The major drawbacks of these apparatuses were that because the electric fields were not uniform, and the angle between the electric field varied across the gel and DNA molecules migrated at different rates depending on their location in the gel. This is especially problematic in mammalian genome mapping, where a continuous distribution of fragment sizes is generated. Lane-to-lane comparisions and size estimations for digested genomic DNA are less straightforward when fewer discrete bands are being separated, as with the chromosomes of lower organisms like yeast. The angle between the electric fields varies from less than 180° and the more than 90°. DNA molecules from 1,000 to 2,000 kb can be separated in OFAGE.

Rotating Gel Electrophoresis (RGE): In England in 1987, Southern described a novel PFGE system that rotates the gel between two set angles while the electrodes are off. In RGE, the electric field is uniform and bands are straight because only

one set of electrodes is used. RGE makes it easy to perform time and voltage ramping. It also enables users to study the effects of different angles, and even to vary these, during an experiment-angle ramping. RGE uses a single homogeneous field and changes the orientation of the electric field in relation to the gel by discontinuously and periodically rotating the gel. Switch times are too long in RGE. The DNA molecules from 50 kb to 6,000 kb can be separated by adjusting the frequency of the gel rotation. In addition, the angle of reorientation can be easily altered simply by changing the angle of rotation.

Programmable Autonomously-Controlling Electrodes (PACE): The PACE electrophoresis system offers precise control over all electric field parameters by independent regulation of the voltages on 24 electrodes arranged in a closed contour. The flexibility of the PACE system derives from its ability to generate an unlimited number of electric fields of controlled homogeneity, voltage gradient, orientation and duration. The PACE system can perform all previous pulsed field switching regimens (i.e. FIGE, OFAGE, PHOGE, unidirectional pulsing), as well as generate voltage clamped homogeneous static fields. The PACE system separates DNA fragments from 100 bp to over 6 Mb. The ability to alter the reorientation angle between the alternating fields permits an in creased speed of separation for large DNA molecules. A computer-driven system known as PACE, designed by Lai *et al.* may be the ultimate PFGE device. It is an extremely useful tool for studying variables such as pulse time, temperature, agarose concentration, voltage and angles between fields affecting DNA migration in PFGE.

Pulsed-Homogeneous Orthogonal Field Gel Electrophoresis (PHOGE): The major difference between this instrument and other gel boxes with homogeneous electric fields is that the field reorientation angle is 90°. PHOGE uses a 90° reorientation angle, but the DNA molecules undergo four reorientations per cycle instead of two. The DNA lanes in PHOGE do not run straight, a phenomenon which has been described for gel

runs involving multiple electric fields in this manner. This system separates DNA fragments of up to 1 Mb.

2.4 RFLP analysis using DNA probes

In this technique, chromosomal restriction digests produced by frequent cutting enzymes are separated by conventional agarose gel electrophoresis, as described above (section 2.3.1), and then the DNA fragments are transferred onto a nitrocellulose or nylon membrane (Fig. 10)(Sambrook *et al.*, 1989). The DNA on the membrane then is hybridized with a specific chemically or radioactively labeled piece of DNA or RNA (a probe), with binds to the relatively few fragments on the membrane that have complementary nucleic acid sequences. Variations in the number and size of the fragments detected by hybridization are referred to as RFLPs.

One common typing method that uses chromosomal DNA preparations and a ribosomal RNA probe is ribotyping. Because all bacterial isolates have one or more chromosomal rRNA operons distributed around the chromosome, and because those sequences are highly conserved, essentially all bacterial isolates can be typed using probes directed to the DNA sequences that encode the rRNA loci using a single rRNA probe. However, enthusiasm for this system has diminished because the approach has proven to be only moderately discriminatory.

2.5. Typing methods using PCR

Polymerase chain reaction, which has been used for several years for the direct detection of many types of infectious agents in clinical samples, has been adapted for use as a typing tool. The hallmark of PCR is the ability to produce literally millions of copies of a particular DNA segment with high fidelity within 3 to 4 hours' time. The procedure requires template DNA (or RNA if a reverse transcriptase step is used initially),

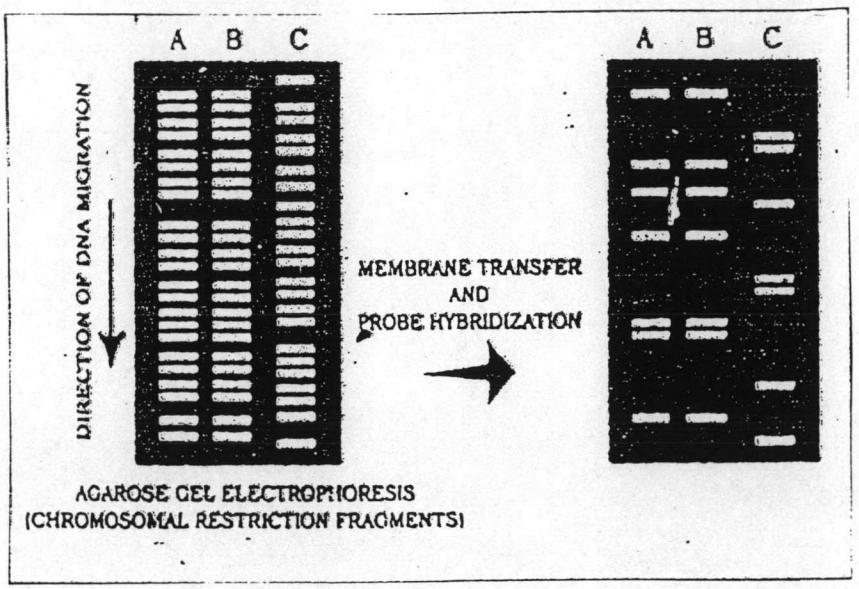

Figure 10. Schematic drawing of restriction fragment-length polymorphism analysis using a DNA or RNA probe, such as IS6110 or ribosomal RNA. The box on the left represents agarose gel electrophoresis of chromosomal DNA cleaved with a restriction endonuclease, and the box on the right represents the nylon filter to which the DNA has been attached and hybridized with a specific probe. Only the DNA fragments on the nylon filter that bind the probe can be visualized. The organisms represented in lanes A and B are indistinguishable, whereas the isolate represented in lane C is a different strain.

which may be present in the sample in minute quantities; two oligonucleotide primers, which flank the sequences on the template DNA to be amplified (thus defining the starting points for DNA polymerase activity); and a heat-stable DNA polymerase. Efficient amplification is accomplished readily for templates of less than 2,000 base pairs, although templates as large as 35 kb now can be amplified by using newer polymerases. A typical PCR assay requires approximately 3 hours to complete 30 cycles, where each cycle consists of a heat denaturation phase, in which double-stranded DNA is melted into single strands; an annealing phase, in which the primers bind to the target sequences on the single strands; and an extension phase, in which DNA synthesis proceeds from the primers along each strand of the template DNA, thereby generating two new double-stranded copies of the original template. After 30 such cycles, a single initial copy of template DNA theoretically can be amplified to 1 billion copies.

XIII. Pulsed-field gel electrophoresis for separation of large DNA

Manipulating and analyzing DNA are fundamentals in the field of molecular biology. Indeed, separating complex mixtures of DNA into different sized fragments by electrophoresis was a well established technique by the early 1970's.

Typically, DNA was isolated intact and then treated with restriction enzymes to generate pieces small enough to resolve by electrophoresis in agarose or acrylamide. Although this procedure still forms the core of DNA separation and analysis in today's laboratories, the rules of the separation have changed.

In 1984, Schwartz and Cantor described pulsed field gel electrophoresis (PFGE), introducing a new way to separate DNA. In particular, PFGE resolved extremely large DNA for the first time, raising the upper size limit of DNA separation in agarose from 30-50 kb to well over 10 Mb (10,000 kb).

After this initial report, a succession of papers described new and improved instrumentation and methods. As a result, routine procedures and several commercial pulsed field units are currently available. Now, instead of cloning a large number of small fragments of DNA, PFGE permits cloning and analysis of a smaller number of very large pieces of a genome.

Applications

Applications of PFGE are numerous and diverse (Gemmill, 1991; Birren and Lai, 1990, 1993; and Van Daelen and Zabel, 1991). These include cloning large plant DNA using yeast artificial chromosomes (YAC's) (Ecker, 1990; and Butler, *et al.*, 1992) and P1 cloning vectors identifying restriction fragment length polymorphisms (RFLP's) and construction of physical maps; detecting *in vivo* chromosome breakage and degradation (Elia, *et al.*, 1991); and determining the number and size of chromosomes ("electrophoretic karyotype") from yeasts, fungi, and parasites such as Leishmania, Plasmodium, and Trypanosoma.

Theory

Bacterial genome is digested with a restriction enzyme that has relatively few recognition sites and thus generates approximately 10 to 30 restriction fragments ranging from 10 to 800 kb. Essentially all of these fragments can be resolved as a pattern of distinct bands by PFGE, using a specially designed chamber that positions the agarose gel between three sets of electrodes that from a hexagon around the gel (CHEF), is applied first in one direction from one set of electrodes, then shifts to the second set of electrodes for a short priod of time (a pulse), and then shifts to the third set of electrodes. Thus, the electric field that causes the DNA to migrate in the gel is provided in pulses that alternate from three sets of electrodes.

Although the theory of pulsed field electrophoresis is a matter of debate, qualitative statements can be made about the movement of DNA in agarose gels during PFGE. During continuous field electrophoresis, DNA above 30-50 kb migrates with the same mobility regardless of size. This is seen in a gel as a single large diffuse band. If, however, the DNA is forced to change direction during electrophoresis, different sized fragments within this diffuse band begin to separate from each other.

With each reorientation of the electric field relative to the gel, smaller sized DNA will begin moving in the new direction more quickly than the larger DNA. Thus, the larger DNA lags behind, providing a separation from the smaller DNA.

Currently, there are three models that attempt to describe the behavior of DNA during PFGE (Chu, 1990), the biased reptation model (BRM), the chain model, and, most recently, the bag model (Chu, 1990, 1991).

Instrumentation

Although many types of PFGE instrumentation are available (fig. 11), they generally fall into two categories. The simplest equipment is designed for field inversion gel electrophoresis (FIGE) (Carle *et al.*, 1986). FIGE works by periodically inverting the polarity of the electrodes during electrophoresis. Because FIGE subjects DNA to a 180° reorientation, the DNA spends a certain amount of time moving backwards. Only an electrical field switching module is needed; any standard vertical or horizontal gel box that has temperature control can be used to run the gel.

Although more complex in its approach, zero integrated field electrophoresis (ZIFE) (Turmel *et al.*, 1990) also falls into this first category. Compared with simple FIGE, ZIFE is very slow. However, ZIFE is capable of resolving larger DNA and giving a larger useful portion of the gel.

The other category contains instruments that reorient the DNA at smaller oblique angle, generally between 96 and 120°. This causes DNA to always move forward in a zigzag pattern down the gel. For a similar size range under optimal conditions, these separations are faster, resolve a wider size range, and give a larger useful portion of the gel compared to FIGE.

Contour-clamped homogeneous electric field (CHEF) (Chu *et al.*, 1986, 1990); transverse alternating field electrophoresis (TAFE) (Gardiner *et al.*, 1986) and its relative ST/RIDEtm (Stratagene); and rotating gel electrophoresis (RGE) (Southern *et al.*, 1987; Anand and Southern, 1990; Gemmill, 1991; and Serwer and Dunn, 1990) are all examples of commonly used transverse angle reorientation techniques for which instrumentation is available. In a further elaboration of the above procedures, Lai and coworkers developed the programmable autonomously controlled electrophoresis (PACE) unit which allows complete control over reorientation angle, voltage, and switch time (Clark, *et al.*, 1988; and Birren, *et al.*, 1989). In contrast with FIGE, these systems require both a special gel box with a specific electrode and gel geometry, and the associated electronic control for switching and programming the electrophoresis run.

Ideally, the DNA should separate in straight lanes to simplify lane-to-lane comparisons. The original pulsed-field systems used inhomogeneous electric fields that did not produce straight lanes, making interpretation of gels difficult (Schwartz and Cantor, 1984). Again, the simplest approach to straight lanes is FIGE, which uses parallel electrodes to assure a homogeneous electric field.

Although extremely useful for separating relatively small DNA, 4- 1,000 kb (fig. 12), FIGE's reorientation angle of 180° results in a separation range most useful under 2,000 kb. Furthermore, like other PFGE techniques, FIGE has mobility inversions in which larger DNA can move ahead of smaller DNA during electrophoresis.

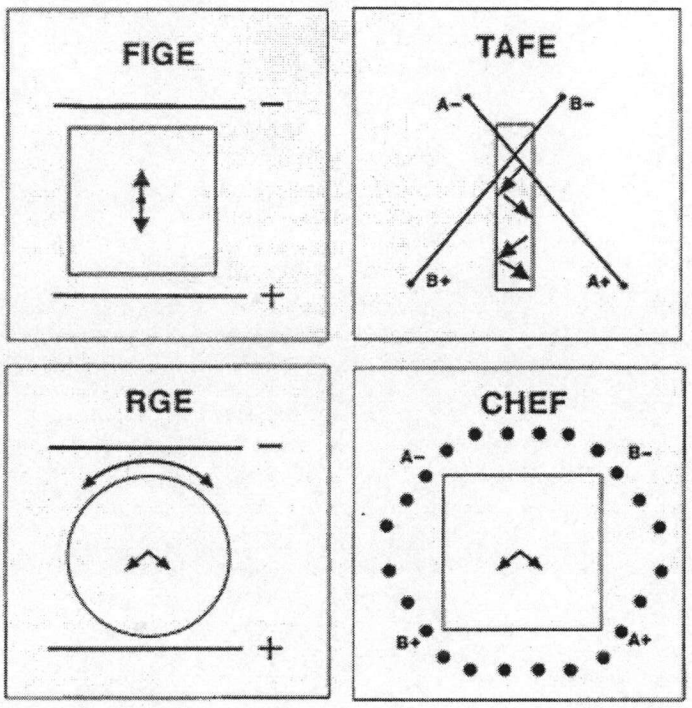

Figure 11. Electrode configuration of commonly used pulsed field gel electrophpresis units.

Ramping, where the reorientation pulse length is constantly increased during separation, will minimize inversions. This capability is included in most commercial instrumentation.

Increasing both the separation range and the resolution of large DNA requires smaller reorientation angles, generally 96-140°, with 120° most common. Smaller angles (e.g., 100°) increase the mobility of the DNA generally without seriously affecting resolution. The lower limit is approximately 96°. Below this, separation is seriously compromised.

TAFE and ST/RIDEtm use a complicated geometry between the electrodes and a vertically placed gel to get straight lanes. CHEF and RGE maintain a homogeneous electric field in combination with a horizontal gel. CHEF changes the direction of the electric field electronically to reorient the DNA by changing the polarity of an electrode array. With RGE the electric field is fixed; to move the DNA in a new direction, the gel simply rotates.

Rotating Gel Electrophoresis

RGE is one of the most recent commercial introductions of pulsed field equipment and combines variable angles with a homogeneous electric field (fig. 13 and 14) (Southern *et al.*, 1987; Anand and Southern, 1990; Serwer and Dunn, 1990; and Gemmill, 1991). The electrodes are positioned along opposite sides of the buffer chamber with their polarity fixed. Briefly, the gel is cast on a circular running plate and then placed in the buffer chamber. The gel is coupled to a magnetic drive beneath the buffer chamber to eliminate the possibility of leakage that a direct connection might cause.

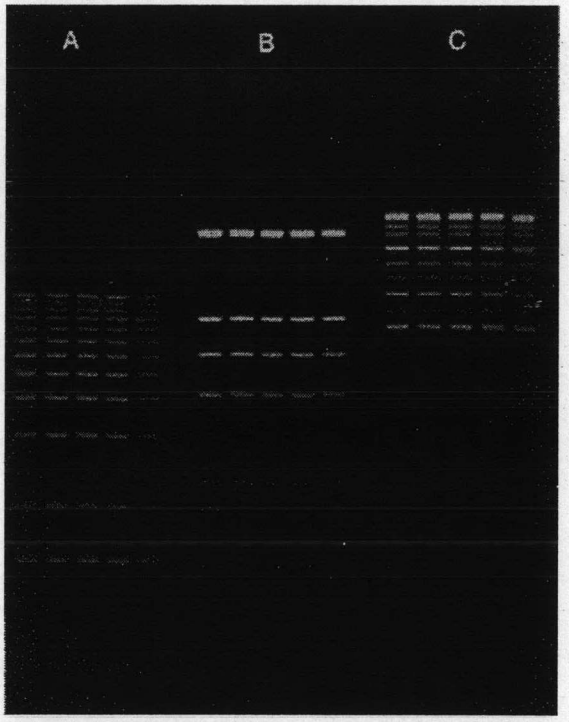

Figure 12. Increased separation of the 20-50 kb range with field inversion gel electrophoresis (FIGE). Run conditions: 230 V, 7.9 V/cm, 16 hrs., 50 msec. pulse, forward:reverse pulse ratio = 2.5:1, 1% GTG agarose, 0.5X TBE, 10 C.a) 1 kb ladder, 0.5-12 kb; b) Lambda/Hind III, 0.5-23 kb; and c) High molecular weight markers, 8.3-48.5 kb.

To force the migrating DNA to a new direction, the magnetic drive simply rotates the gel to the new angle. Because the reorientation angle of the DNA is determined by a straightforward mechanical coupling, RGE offers a lot of flexibility at a reduced cost. Voltage, angle, and pulse times are varied with the program stored into memory of the unit.

Sample Preparation

Along with the ability to separate large DNA came the need for new sample preparation and handling procedures. Large DNA (e.g., yeast chromosomes) is easily sheared and also difficult to pipet due to its high viscosity. The solution to this problem is to first embed the bacteria or yeast in agarose plugs and then treat the plugs with enzymes to digest away the cell wall and proteins, thus leaving the naked DNA undamaged in the agarose. The plugs then are cut to size, treated with restriction enzymes if necessary, loaded in the sample well, and sealed into place with agarose.

Choice of restriction enzyme

The choice of restriction enzyme(s) is influenced by several factors. The first is the G+C content of the bacterial species: DNA of low G+C (e.g., *S. aureus* DNA) will cut infrequently when treated with restriction enzymes with a G+C-rich recognition site (e.g., *Sma* I). Second, enzymes with 8-bp recognition sequences will cut less frequently than comparable 6-bp cutters (Maslow *et al.*, 1993).

Separation Area

Most PFGE systems separate DNA over a relatively small area, limiting the resolution of complex samples. RGE is an exception to this, with a useful separation distance up to 20 cm and a maximum gel size of 18 x 20 cm.

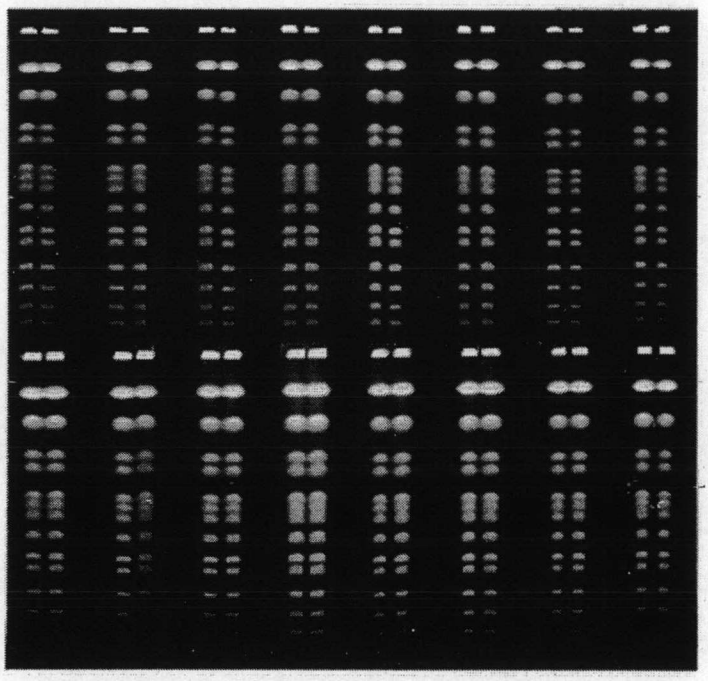

Figure 13. Rotating gel electrophoresis (RGE) separation *Saccharomycescercevisiae* chromosomes (245-2190 kb). Run conditions: 180 V, 5.1 V/cm, 34 hrs., 120 angle, 60-120 sec. pulse ramp, 0.5X TBE, 1.2% GTG agarose, 10°C. Two combs were used on the same gel to load 32 samples, a maximum of 72 are possible

Separation Parameters

Several parameters act in concert during PFGE (Southern *et al.*, 1987; Anand and Southern, 1990; Birren *et al.*, 1989; and Gemmill, 1991). These will be discussed briefly below as they relate to transverse field instruments such as RGE. The minimum amount of information needed to repeat a separation should include a short description of the pulsed field instrumentation used; applied voltage and field strength (e.g., 180 V at 5.3 V/cm); pulse length (e.g., 87 seconds); reorientation angle (e.g., 120°); the buffer (0.5X TBE); the agarose type and concentration (SeaKem Gold, 1.1%); the buffer chamber temperature (e.g., 10°); the type of standards (Clontech *S. cerevisiae*); and, if possible, the amount of DNA loaded. Although the data listed above is necessary to faithfully reproduce a separation, the information supplied in publications is rarely this complete.

Field Strength

The field strength has a profound effect on pulsed field separations and is a compromise between separation time and resolution of a particular size class. Four to six volts/cm is generally required for resolving DNA up to 2000 kb (e.g., *S. cerevisiae* chromosomes) in a reasonable period of time (e.g., 1-2 days). However, these field strengths trap and immobilize even bigger DNA in the agarose matrix, and DNA > 3000 kb requires 2 V/cm or less for separation.

Pulse Time

Pulse time primarily changes the size range of separation. Longer pulse times lead to separation of larger DNA. For example, at 5.4 V/cm, the 1.6 Mb and 2.2 Mb chromosomes from *S. cerevisiae* separate as a single band with 90-second pulse length. Increasing the pulse length to 120 seconds resolves these into two bands (Gemmill, 1991).

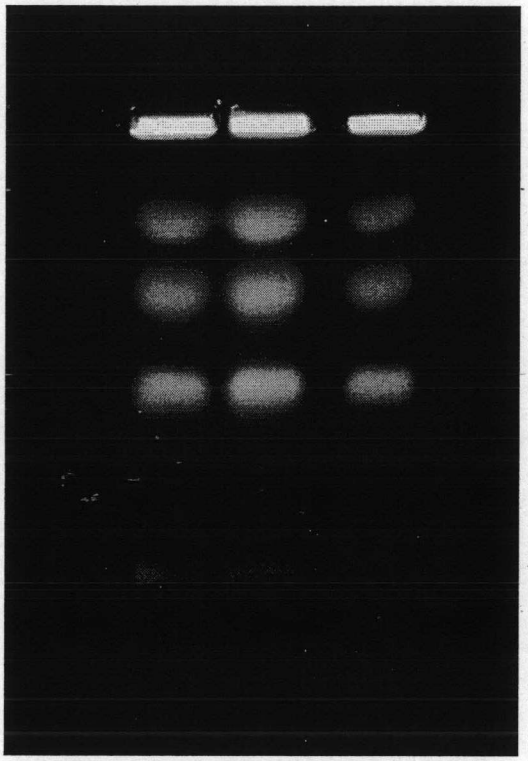

Figure 14. Rotating gel electrophoresis (RGE) separation of 3000 to 6000 kb DNA *Schizosaccharomyces pombe* chromosomes. Run conditions: 50 V, 1.4 V/cm, 100 hrs, 100 angle, concatamated multiple runs: 2500 sec./50hrs, 3000 sec./50hrs, 0.5X TBE, 0.8% megarose (Clontech), 10 °C.

Reorientation Angle

Any angle between 96 and 165° produces roughly equivalent separation (Birren *et al.*, 1988; and Gemmill, 1991). The smaller the angle, however, the faster the DNA mobility. And for separating extremely large DNA, 96 to 105° is almost a requirement to get a good separation in the shortest possible time.

Buffers

Two buffers are commonly employed for PFGE--TAE and TBE (1x TAE is 40 mM Tris acetate, 1 mM EDTA, pH 8.0; 1x TBE is 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). Both are used at a relatively low ionic strength to prevent heating and carry the designations of either 0.25 and 0.5x to indicate the dilution relative to the standard concentration. An added benefit to low ionic strength buffers is an increase in DNA mobility. For example, while using RGE to compare various buffers and agaroses, found that lowering both TAE and TBE to 0.25X gave the maximum mobility (40-50% faster than 1x). Below 0.25X, the DNA mobility dropped off (White, 1992).

Agarose

The type of agarose also affects DNA separation, with the fastest mobilities and best resolution achieved in gels made of low electroendosmosis (EEO) agarose (Birren *et al.*, 1989; and White, 1992). Although most standard electrophoresis grades of agarose are suitable for PFGE (e.g., SeaKem GTG), agarose with minimal EEO will provide a faster separation. Several low EEO "pulsed field grades" are available, including FastLane and Gold (FMC BioProducts), and Megarose (Clontech).

The concentration of agarose affects both the resolution and mobility of the DNA (Birren *et al.*, 1989; and White, 1992). Higher concentrations of agarose yield sharper, but

slower moving bands. And the typical concentrations used (0.8-1.2%) represent a tradeoff between speed and resolution. High percentages of low EEO agarose may improve resolution without sacrificing the speed of separation (White, 1992).

Temperature

Because DNA mobility also depends on the separation temperature, the temperature must be constant both during and between runs. Although higher temperatures increase DNA mobility, it does so at the expense of resolution (Birren *et al.*, 1989; and Gemmill, 1991).

PFGE has evolved into a routine, pragmatic technique for molecular biologists. This is reflected in the present availability of methods chapters and manuals (e.g., Birren and Lai, 1990, 1993; Anand and Southern, 1990; Daelen and Zabel, 1991).

What does the future hold? Possibilities include using a new or improved separation material, and going beyond the current size limit of > 10 Mb. Anecdotal reports suggest separations in the range of 20 Mb or larger are possible, which would further simplify the complex task of genome mapping.

XIV. Interpreting Chromosomal DNA Restriction Patterns Produced by Pulsed-Field Gel Electrophoresis: Criteria for Bacterial Strain Typing.

To determine the relatedness of a group of bacterial isolates, that is, to type them. During the last decade, traditional methods of strain typing, such as bacteriophage typing and serotyping, have been supplemented or replaced in many laboratories with newer molecular methods, such as plasmid fingerprinting (Tenover, 1985), ribotyping (Stull *et al.*, 1988), PCR-based methods (Belkum, 1994), and analysis of chromosomal DNA restriction patterns by pulsed-field gel electrophoresis(PFGE) (Arbeit *et al.*, 1990; Finney,

1993; and Maslow *et al.*, 1993). Although bacteriophage typing is still used in a number of large reference laboratories around the world for epidemiologic studies of *Staphylococcus aureus* (Schlichting *et al.*, 1993) and serotyping continues to be a useful tool for epidemiologic surveillance of *Salmonella* species (Olsen *et al.*, 1994), there is a need for a method of strain typing that can be used to type a broader array of bacterial species. At present, PFGE comes closest to satisfying that need (Arbeit, 1995; and Swaminathan and Matar, 1993).

PFGE involves embedding organisms in agarose, lysing the organisms in situ, and digesting the chromosomal DNA with restriction endonucleases that cleave infrequently (Finney, 1993; and Maslow *et al.*, 1993). Slices of agarose containing the chromosomal DNA fragments are inserted into the wells of an agarose gel, and the restriction fragments are resolved into a pattern of discrete bands in the gel by an apparatus that switches the direction of current according to a predetermined pattern. The DNA restriction patterns of the isolates are then compared with one another to determine their relatedness. Currently, there are no standardized criteria for analyzing the fragment patterns. Consequently, different investigators viewing the same PFGE results may come to quite different conclusions as to which isolates should be designated as outbreak related and which should be designated as non-outbreak related. This guest commentary proposes a set of guidelines for interpreting DNA restriction patterns generated by PFGE. The authors are investigators from the United States who, over the last several years, have correlated epidemiologic data from dozens of outbreaks with strain typing results produced by PFGE. These guidelines are intended to be used by clinical microbiologists in hospital laboratories to examine relatively small sets of isolates (typically, $\leq$30) related to putative outbreaks of disease. In an effort to make PFGE more easily understood and accessible as a typing method, the use of statistical methods and equipment to digitize patterns has been avoided. Such methods may be appropriate for larger collections of isolates studied in

reference laboratories, but they are neither feasible nor necessary for laboratories that will be confronted primarily with short-term outbreaks.

Definitions

The following vocabulary will be used throughout this commentary.

Isolate: Isolate is a general term for a pure culture of bacteria obtained by subculture of a single colony from a primary isolation plate, presumed to be derived from a single organism, for which no information is available aside from its genus and species.

Epidemiologically related isolates: Epidemiologically related isolates are isolates cultured from specimens collected from patients, fomites, or the environment during a discrete time frame or from a well-defined area as part of an epidemiologic investigation that suggests that the isolates may be derived from a common source.

Genetically related isolates (clones): Genetically related isolates (clones) are isolates that are indistinguishable from each other by a variety of genetic tests (e.g., PFGE, multilocus enzyme electrophoresis, or ribotyping) or that are so similar that they are presumed to be derived from a common parent. (Given the potential for cryptic genetic changes detectable only by DNA sequencing or other specific analyses, evidence for clonality is best considered relative rather than absolute [Eisenstein, 1989]).

Outbreak: An outbreak is the increased incidence of an infectious disease in a specific place during a given period that is above the baseline rate for that place and time frame.

Strain: A strain is an isolate or group of isolates that can be distinguished from other isolates of the same genus and species by phenotypic characteristics or genotypic characteristics or both. A strain is a descriptive subdivision of a species.

Outbreak strain: Outbreak strains are isolates of the same species that are both epidemiologically related (e.g., by time, place, and common source of infection) and genetically related (i.e., have indistinguishable genotypes). Such isolates are presumed to be clonally related since they have common phenotypes and genotypes and were isolated within a defined period.

Endemic strain: Endemic strains are isolates that are recovered frequently from infected patients in a particular health care setting or community and that are indistinguishable or closely related to each other by typing methods but for which no direct or epidemiologic linkage can be demonstrated. Such organisms are presumed to be clonally related, but their common origin may be more temporally distant from those of outbreak strains.

Assumptions for strain typing

The goal of strain typing studies is to provide laboratory evidence that *epidemiologically related* isolates collected during an outbreak of disease are also *genetically related* and thus represent the same strain. This information is helpful for understanding and controlling the spread of disease in both hospitals and communities. The use of strain typing results for infection control decisions is based on several assumptions: (i) isolates representing the outbreak strain are the recent progeny of a single (or common) precursor, (ii) such isolates will have the same genotype, and (iii) epidemiologically unrelated isolates will have different genotypes. By chance, some epidemiologically unrelated isolates may have similar or indistinguishable genotypes, particularly if there is limited genetic diversity within a species or subtype (Barrett *et al.*, 1995). For example, most strains of methicillin-resistant *S. aureus* are derived from a small number of ancestral clones (Kreiswirth *et al.*, 1993; Schlichting *et al.*, 1993; and Tenover *et al.*, 1994). Thus, when methicillin-resistant *S. aureus*, which is a common

nosocomial pathogen, is endemic in a hospital, it can be difficult to discern when an outbreak of methicillin-resistant *S. aureus* occurs, especially if the endemic strain is responsible. In practice, typing is most effective as an aid to outbreak investigations when it is applied to small sets of isolates that are epidemiologically related. The isolates should be obtained from patients, fomites, and environmental sources that are related to (i) the area in which infections are occurring, (ii) the period during which the infections occurred, and (iii) a common source of infection. If possible, typing studies should be performed in a blinded fashion to reduce bias. Typing studies performed on isolates for which epidemiologic information is not and will not be available may produce misleading information. Strain typing data do not substitute for epidemiologic data. Rather, the two data sets should be developed independently but analyzed together to determine whether an outbreak has occurred.

The interpretive criteria

To interpret the DNA fragment patterns generated by PFGE and transform them into epidemiologically useful information, the microbiologist must understand how to compare PFGE patterns and how random genetic events can alter the patterns. Ideally, the PFGE patterns of isolates representing the outbreak strain would be indistinguishable from each other and distinctly different from those of epidemiologically unrelated strains. When this occurs, the outbreak strain is easy to identify (Haley *et al.*, 1995). More commonly, random genetic events, including point mutations and insertions and deletions of DNA, alter PFGE patterns during the course of an outbreak. While this makes interpretation of the patterns a little more challenging, knowledge of how such genetic events affect the patterns enables the microbiologist to correctly assign the pattern of each isolate to one of four categories: indistinguishable from the outbreak pattern, closely related to the outbreak pattern, possibly related to the outbreak pattern, or unrelated to the outbreak pattern. The

criteria proposed herein are reliable if PFGE resolves at least 10 distinct fragments. When fewer bands are detected, the robustness and discriminatory ability of the criteria are unknown. We believe that the comparison of restriction patterns remains, in part, a subjective process that cannot be totally reduced to rigid algorithms. However, the process becomes easier and more consistent with experience. As noted earlier, although equipment to perform computerbased image acquisition and analysis is available, such equipment is not widely available in clinical laboratories, and thus, the use of such equipment has not been incorporated into these criteria.

Analyzing the restriction patterns and assigning the isolates to categories of relatedness

First, examine the patterns to identify the common or outbreak pattern, which is presumed to represent the pattern for the outbreak strain. If there is no common pattern, then the isolates are most likely unrelated. (Among epidemiologically related isolates, the absence of a common pattern is a rare event.) After identifying the outbreak pattern, the size and number of the fragments in the outbreak pattern are compared with the fragments that make up the patterns of the other isolates. On the basis of pairwise, fragment-for-fragment comparisons, each isolate's pattern is then classified for its relatedness to the outbreak pattern. Patterns that are distinctly different from the outbreak pattern (fewer than 50% fragments in common) are considered unrelated types. Patterns that differ from the outbreak pattern by two or three fragment differences (as described below) are considered to be subtypes of the outbreak pattern. The various restriction pattern changes are illustrated in figure 15 and are summarized in table 9. The criteria for interpreting PFGE patterns are summarized in table 10 and are discussed in detail below.

Categories of genetic and epidemiologic relatedness

Indistinguishable: Isolates are designated genetically indistinguishable if their restriction patterns have the same numbers of bands and the corresponding bands are the same apparent size (fig. 15, lane A). The epidemiologic interpretation of these results is that the isolates are all considered to represent the same strain; i.e., isolates demonstrating the common outbreak pattern represent the outbreak strain. For many species, comparative studies indicate that isolates that are indistinguishable by PFGE are unlikely to demonstrate substantial differences by other typing techniques (Gordillo *et al.*, 1993; Miranda *et al.*, 1991; Olsen *et al.*, 1994; Schoonmaker *et al.*, 1992; Struelens *et al.*, 1992; and Tenover *et al.*, 1994).

Closely related: An isolate is considered to be closely related to the outbreak strain if its PFGE pattern differs from the outbreak pattern by changes consistent with a single genetic event, i.e., a point mutation or an insertion or deletion of DNA. Such changes typically result in two to three band differences (fig. 15; table 9). For example, a spontaneous mutation that creates a new chromosomal restriction site (a single genetic event) will split one restriction fragment into two smaller fragments. (The sum of the sizes of the two smaller fragments should approximate the size of the larger fragment.) The loss of the original large fragment is a one-band difference, and the appearance of two new smaller fragments represents two additional band differences; thus, there is a three bands difference between the outbreak pattern and that of the test isolate. Such an isolate is considered to be closely related to the outbreak strain because by PFGE analysis they differ by only a single genetic event (table 10). Variations of two to three bands have been observed in strains of some species when they are cultured repeatedly over time or isolated multiple times from the same patient (Arbeit *et al.*, 1993; and Sader *et al.*, 1993).

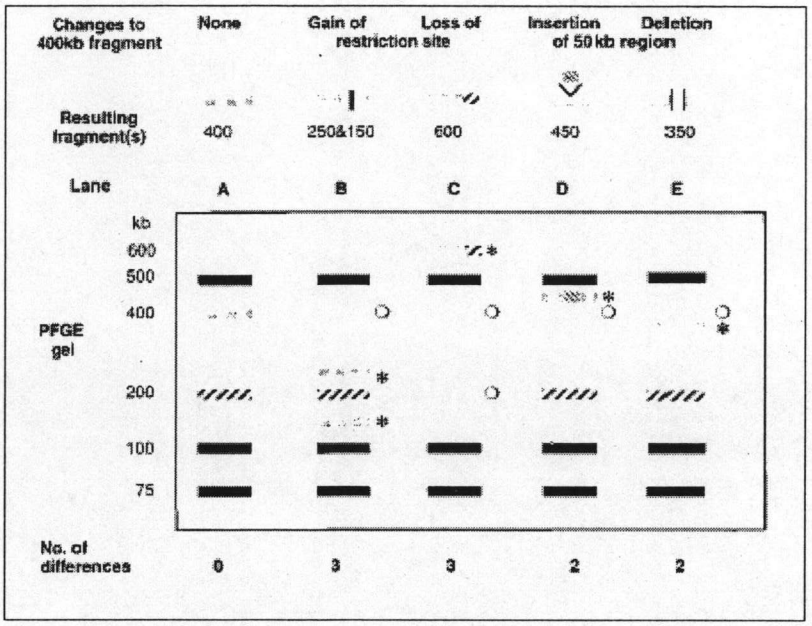

Figure 15. Schematic diagram showing the changes in the PFGE pattern of an isolate as a result of various genetic events. Lane A, outbreak pattern; lane B, gain of a restriction site; lane C, loss of a restriction site; lane D, insertion of DNA in an existing fragment; lane E, deletion of DNA from an existing fragment. The open circles indicate fragments present in the outbreak pattern and missing from the test isolate after a genetic event; asterisks indicate fragment present after a genetic event but absent from the outbreak pattern.

Possibly related: An isolate is considered to be possibly related to the outbreak strain if its PFGE pattern differs from the outbreak pattern by changes consistent with two independent genetic events (i.e., four to six band differences that can be explained by simple insertions or deletions of DNA or the gain or loss of restriction sites). While these isolates may have the same genetic lineage as that of the outbreak strain, they are not as closely related genetically and, consequently, are less likely to be related epidemiologically (Table 10). Such variation has been observed among isolates collected over longer periods ($\geq$6 months) or taken from large numbers of patients involved in extended outbreaks. Isolates that are possibly related genetically but that have no epidemiologic link to the outbreak strains are likely to differ by other typing techniques, such as plasmid fingerprinting (Arbeit, 1995; and Tenover *et al.*, 1994).

Unrelated: An isolate is considered unrelated to the outbreak strain if its PFGE pattern differs from the outbreak pattern by changes consistent with three or more independent genetic events (generally seven or more band differences). Typically, this implies that ,50% of the well-resolved fragments present in the pattern from such an isolate will be present in the outbreak pattern.

Method of reporting: The DNA restriction pattern that is designated the outbreak pattern is usually reported as type A; the isolates whose restriction patterns are indistinguishable from that pattern are reported as representing the outbreak strain. Patterns that are closely or possibly related to the outbreak pattern are considered subtypes of A and are designated type A1, type A2, etc. Isolates with closely or possibly related restriction patterns are reported as probably or possibly epidemiologically related, respectively. Patterns that differ substantially from the outbreak pattern and that are classified as unrelated are designated type B, type C, etc. Isolates with unrelated patterns are considered unrelated epidemiologically.

Table 9. Effects of genetic events on PFGE fragment patterns.

Type of genetic event	**Resulting changes in PFGE pattern compared with the outbreak pattern**
Point mutation resulting in creation of a restriction site	The altered pattern will lack one fragment present in the outbreak pattern and, concomitantly, will have two new smaller fragments not present in the outbreak pattern; the sum of the sizes of the two smaller fragments should approximate the size of the larger fragment. This is considered a three-fragment difference (Fig. 15, lane B).
Point mutation resulting in loss of a restriction site	The altered pattern will have a new larger fragment not present in the outbreak pattern and will lose two smaller fragments. This is a three-fragment difference (Fig. 15, lane C).
Insertion of DNA into an existing restriction fragment (new DNA does not have a restriction site)	The altered pattern will have the same number of fragments as the outbreak pattern, but it will lack one small fragment and will show a new fragment of a larger size. This two-fragment difference is commonly referred to as a fragment shift (Fig. 15, lane D).
Deletion of DNA from a fragment (deleted material does not contain a restriction site)	The altered pattern will show a new fragment of a smaller size and loss of a larger fragment. This is a two-fragment difference (Fig. 15, lane E).

Table 10. Criteria for interpreting PFGE patterns

Interpretation based on typing results	**No. of genetic differences compared with outbreak strain**	**Typical no. of fragment differences compared with outbreak pattern**	**Epidemiologic interpretation**
Indistinguishable	0	0	Isolate is part of the outbreak
Closely related	1	2-3	Isolate is probably part of the outbreak
Possibly related	2	4-6	Isolate is possibly part of the outbreak
Different	≥3	≥7	Isolate is not part of the outbreak

Before PFGE can be considered reliable for typing a given bacterial species, the technique must be validated by demonstrating that when restriction fragment patterns are analyzed by PFGE, epidemiologically unrelated isolates generate unique fragment patterns and epidemiologically related isolates generate indistinguishable or, on occasion, closely related fragment patterns. Discriminatory power and reproducibility are important attributes in any typing system. For most of the common bacterial pathogens, the validity of PFGE for molecular typing is well established (Arbeit, 1995).

Controls

A well – characterized control strain should be processed along with the unknown isolates being tested. Obtaining expected results with the control organism affirms that (i) the procedure, including the cell lysis, washing, and endonuclease digestion steps, is working; (ii) the gel and electrophoretic conditions have been appropriate; and (iii) the conditions of the procedure have yielded results that are reproducible from run to run within the laboratory and that are consistent with those obtained by other investigators for the same strain.

A molecular size standard should be run in at least one lane of the gel to provide size orientations of the fragments. (It is often helpful to run standards in one outside lane and in a lane in the middle of the gel.) Standards are needed to evaluate minor profile differences that may result from single genetic events such as deletions, insertions, or mutations. The molecular sizes of unknown fragments can be determined by plotting the distance of migration (in millimeters from the bottom of the sample well in the gel) of the standard against the log10 of the molecular size of the fragments. This plot can be used to convert the migration distance of the bands in the test samples to molecular size. In most cases, visual estimates of fragment sizes are adequate for interpretation of PFGE profile differences, and it is not necessary to calculate the sizes of the fragments for the test

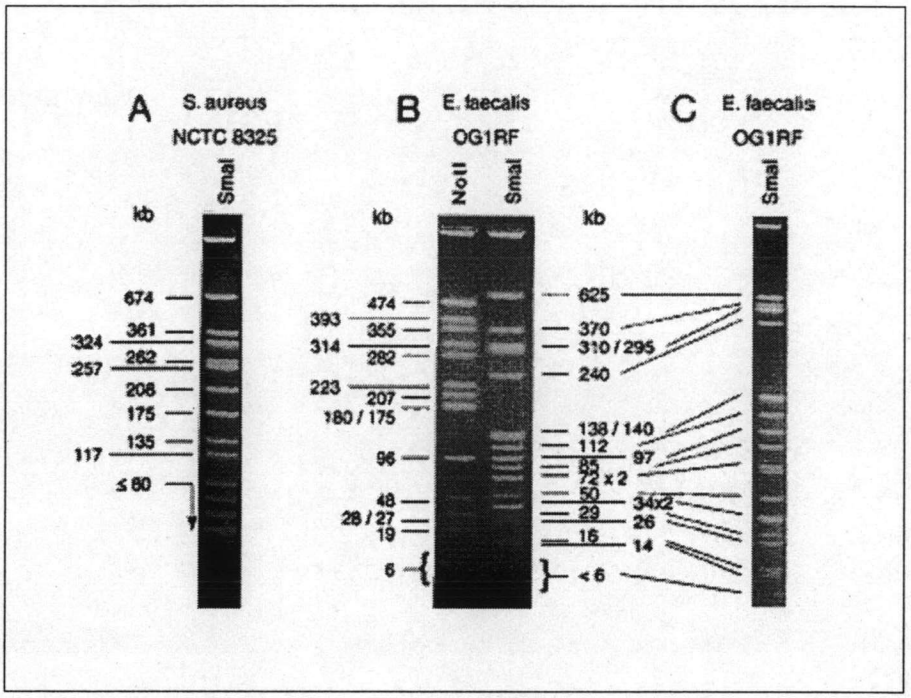

Figure 16. PFGE patterns of chromosomal DNA restriction fragments resolved in 1.6% Seakem Gold agarose in 0.53 TBE buffer (35) for *S. aureus* NCTC 8325 DNA digested with *Sma*I (pulse times, 2 to 45 s; running time, 29.5 h) (A), *E. faecalis* OG1RF DNA digested with *Not*I or *Sma*I (pulse times, 2 to 45 s; running time, 29.5 h) (B), and *E. faecalis* OG1RF DNA digested with *Sma*I (pulse time, 2 to 21 s; running time, 50 h) (C). The sizes of the fragments are indicated in kilobases.

strains. Preparations consisting of phage lambda concatamers, referred to as a "lambda ladder," are commonly used as molecular size standards, and some vendors offer preparations that contain an enhanced 48-kb band, which is helpful for size orientation in the gel. Other preparations containing fragments of known size, such as agarose plugs containing restriction endonuclease-digested *Saccharomyces cerevisiae* DNA, are also commercially available, but they are not needed for determining the sizes of bands obtained in most PFGE strain typing protocols. The DNA restriction fragment patterns of several well-characterized organisms, in which the size of each chromosomal fragment has been determined, are shown in (Fig. 16 and 17). These include patterns for *S. aureus* NCTC 8325, *Enterococcus faecium* GE1 (ATCC 51558), *Escherichia coli* MG1655 (ATCC 47076), and *Enterococcus faecalis* OG1RF (ATCC 47077). Inclusion of one of these strains in each group of isolates to be tested provides both a procedure control and a molecular size standard.

When typing a set of isolates suspected of being part of an outbreak, it is helpful to include a sample of epidemiologically unrelated isolates as well to ensure that endemic strains can be differentiated from outbreak strains. This is particularly important for analyzing outbreaks of methicillin-resistant *S. aureus* in which the overall number of PFGE patterns is limited (Schlichting *et al.*, 1993; and Struelens *et al.*, 1992).

Safety

Three areas of safety that need to be considered before applying PFGE to pathogenic bacteria are the hazards associated with propagating the bacteria, handling and disposing of chemical reagents, and electrical hazards.

Before they are embedded in agarose plugs, bacteria are grown in 1 to 10 ml of broth to the late-log or stationary phase, centrifuged, washed, and adjusted to the appropriate cell density in a buffer. Precautions generally used to prevent aerosols during

these steps need to be followed, e.g., centrifugation in appropriate containers fitted with leak-proof caps. Also, precautions to prevent the release of the infectious agent by breakage of tubes, spills, and aerosolization must be used during vortex mixing of the bacteria to resuspend them in buffer (Richmond and McKinney, 1993). These precautions should be taken for all bacteria that require handling at Biosafety Level 2 (Richmond and McKinney, 1993); additional precautions may be necessary to handle bacteria that have low infectious doses (e.g., *Shigella* sp. and *Escherichia coli* O157:H7) or those known to cause laboratory-acquired infections, particularly via the respiratory route.

After the bacteria are embedded in agarose plugs, the mold used to cast the plugs should be soaked in disinfectant. After the chemical lysis step, most gram-negative bacteria embedded in agarose plugs will be inactivated; however, complete killing may not occur for gram-positive spore-forming and non-sporeforming bacteria, including *Mycobacterium tuberculosis*. For these organisms, the efficiency of the lysis procedure for killing the cells should be determined by culturing the plugs after the lysis step.

Two hazardous chemicals may be encountered when performing PFGE. Ethidium bromide, which intercalates between the bases of nucleic acids and fluoresces when it is exposed to UV light, is widely used to stain gels to resolve the fragments for photography. It is a powerful mutagen (Sambrook *et al.*, 1989). Another chemical, phenylmethylsulfonyl fluoride was used in many early PFGE protocols to inactivate proteinase K. It is very destructive to the mucous membranes of the respiratory tract, eyes, and skin and may be fatal if it is inhaled, swallowed, or absorbed through the skin (Sambrook *et al.*, 1989). Recent protocols avoid the use of this reagent (Maslow *et al.*, 1993). Laboratory personnel who use these chemicals should be aware of their hazardous properties and should take appropriate precautions.

The electrophoretic separation of large DNA fragments by PFGE is done at higher voltages (5 to 10 V/cm) than those used for conventional agarose submarine gel

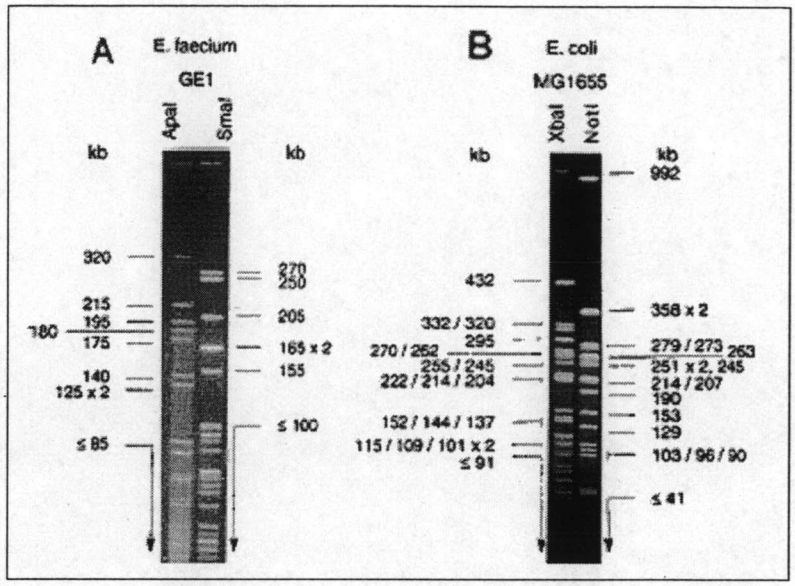

Figure 17. PFGE patterns of chromosomal DNA restriction fragments resolved in 1.6% Seakem Gold agarose in 0.53 TBE buffer (35) for *E. faecium* GE1 DNA digested with *Apa*I or *Sma*I (pulse time, 2 to 21 s; running time, 50 h) (A) and *E. coli* MG1655 DNA digested with *Xba*I or *Not*I (pulse time, 5 to 75 s; running time, 55 h) (B). The sizes of the fragments are indicated in kilobases.

electrophoresis of smaller DNA fragments (1 to 5 V/cm). Commercially available PFGE apparatuses usually have safety interlocks to prevent opening of the apparatus while it is still connected to the power supply. However, to maintain an even temperature throughout the gel during electrophoresis, many systems recirculate the running buffer through a cooling apparatus. Leaks in the recirculation system may expose the operator to high voltages.

XV. Molecular Typing methods of *Salmonella* strain :

Efficienct typing techniques are a prerequisite for effective surveillance of infections caused by members of the genus *Salmonella*. Serotyping which allows differentiation of more than 2,500 different antigenic combinations (Popoff *et al.*, 1991) has provided an excellent basis for epidemiologic investigations if rare serotypes are involved (Holmberg *et al.*, 1984). However, if one particular serotype is responsible for more than just a few percent of cases, additional methods are needed to discriminate strains. Phage typing has been the method of choice foe many years or even decades for further subtyping the most important serotypes (*eg Salmonella* Typhi, *Salmonella* Typhimurium, *Salmonella* Enteritidis). Although this method is applicable to large numbers of strains it has several shortcomings including limited sensitivity and the need to have available all phage suspensions (*eg* 100+ for *Salmonella* Typhi) (Altwegg, 1992). Therefore, newly developed molecular typing methods have also been applied to analyze *Salmonella* isolates. Most of these methods include digestion of chromosomal DNA with restriction enzymes, separation of the resulting fragments by regular agarose or by pulsed-field gel electrophoresis, and staining in ethidium bromide or hybridization with labeled probes (radioactive or non-radioactive).

Plasmid analyzing: Non-chromosomal genetic elements have been used for epidemiological investigations for a long time with enormous success (Mayer, 1988)

despite the fact that plasmids may be quite rare in certain bacterial species and that they may be lost during repeated subculturing. The stability of plasmids *in vitro* depends on storage temperature and on the plasmid to be investigated: under any conditions analyzed the virulence- associated plasmid of *Salmonella* Enteritidis has never been lost, whereas none, 6/450 and 70/440 colonies of 4 *Salmonella* Berta and 1 *Salmonella* Enteritidis strains exhibited some change in plasmid profiles when strains were stored at -80°C, 5°C, and 22°C, respectively, for 2.5 years (Olsen *et al.,* 1994). It is not known wherther this also reflects plasmid stability *in vivo*, however, in an AIDS patient with persistent *Salmonella* Typhimurium infection 4 isolates obtained up to 7 months apart had identical REA-, ribotyping- and IS200 patterns but one out of two plasmids (59 mDa and 38 mDa) was not found consistently (Fica *et al.*, 1994). In a study from southern Switzerland investigating a limited *Salmonella* Typhimurium epidemic, plasmid patterns were found useful and about as accurate as lysotyping (Brunner *et al.*, 1983) whereas in Italy epidemic and non-epidemic strains of *Salmonella* Typhimurium untypable by the phage typing scheme of Anderson were not reliably differentiated using plasmid profiles because about 50% of the strains harbored a single 60 mDa plasmid (Nastasi *et al.,* 1988).

Restriction enzyme analysis: Digestion of high molecular weight chromosomal DNA with restriction enzymes followed by agarose gel electrophoresis and ethidium bromide staining (restriction enzyme analysis, REA) has provided valuable subtyping of strains in various enterobacterial and non-enterobacterial species. For *Salmonella* serotypes, REA paterns seem to remain constant over months *in vivo* (Fica *et al.*, 1994) but they are less discriminatory than patterns obtained after blotting and hybridization with various probes (rDNA, IS200) (Altwegg *et al.*, 1989; and Martinetti and Altwegg, 1990). In *Salmonella* Typhimurium, REA did not reliably separate epidemic from non-epidemic strains (Nastasi *et al.*, 1988).

Ribotyping: Determination of rRNA gene restriction fragments as originally described by Grimont and Grimont, 1986 (*ie* REA followed by blotting to a membrane and hybridization with a probe containing rRNA sequences) has advantages as compared to other molecular typing systems in that it is not restricted to certain species (such as phage typing) or genera (such as IS2000) but rather universally applicable to all bacteria with only minor experimental changes (DNA isolation procedure, choice of restriction enzyme). In addition, it has proven very useful for many different organisms including *Salmonella* Wien (Pignato *et al.*, 1992). *Salmonella* Typhimurium (Nastasi *et al.*, 1993), *Salmonella* Bovismorbificans (Nastasi *et al.*, 1994), *Salmonella* Infantis (Pelkonen *et al.*, 1994) and others, but less so for *Salmonella* Berta. (Olsen *et al.*, 1992) with more than 90% of such strains from Denmark being indistinguishable by ribotyping.

Insertion sequence IS200: This mobile genetic element which can be used instead of rRNA for probing Southern blots is found on conserved loci on the chromosome of many *Salmonella* serotypes but not in other members of the family *Enterobacteriaceae* (Threlfall *et al.,* 1994). It was first described by Lam and Roth (1983). Transposition is rare under laboratory conditions (Casedesus and Roth, 1989) and this stability makes it a promising tool for strain discrimination within serotypes and for phylogenetic analyses. However, probing restriction enzyme digests with IS200 was not sufficiently sensitive for primary discrimination of *Salmonella* Enteritidis, *Salmonella* Typhi, and *Salmonella* Virchow (Torre *et al.*, 1993) all of which are phage typable serotypes. On the other hand, IS200 typing has been considered useful for genotypic subdivision of *Salmonella* Typhimurium (Stanley *et al.*, 1993) and *Salmonella* Heidelberg (Cooperstock *et al.*, 1992). IS200 allows separation of *Salmonella* Paratyphi B and *Salmonella* Java, two biovars of a single serotype causing paratyphoid fever and gastroenteritis, respectively (Ezquerra *et al.*, 1993). No similar separation was achieved by ribotyping. For the common phage type 193

of *Salmonella* Typhimurium IS200 typing seems to be more discriminatory than ribotyping (Baquar *et al.,* 1994).

Pulsed-field gel Electrophoresis: If enzymes which recognize only few restriction sites within a given genome ("rare cutter") are used for digestion of chromosomal DNA, the fragments produced are long and require special techniques for separation (pulsed-field gel electrophoresis, PFGE) involving alternating electric fields. Maslow *et al* (1993) provided rules on how to interpret PFGE results: (i) strains with identical patterns are considered clonal; (ii) strains with band shifts consistent with a single genetic event (point mutations affecting restriction sites, insertions, deletions, inversions) are also considered clonally related. As shown below, these rules might also be applied to other molecular typing methods, *ie* ribotyping and IS200 typing.

Direct amplification fingerprinting: A newly developed strategy that has proven useful for intraspecies discrimination of strains by the use of the polymerase chain reaction and shot (< 10 bases) oligonucleotide primers is the method of random amplification fingerprinting (DAF) or randomly amplified polymorphic DNA (RAPD). Because this method is dependent on many different experimental parameters (temperature profile, brand of cycler, DNA extraction method, etc) only.

Salmonella Enteritidis, plasmid analysis has been shown to be of very limited use for subyping of *Salmonella* Enteritidis despite the fact that at least one plasmid can be found in most strains (Helmuth and Schroeter, 1994). However, the vast majority of strains is characterized by the presence of a common (virulence-associated) plasmid of 38 mDa (55 kb). Additional plasmids that would allow discrimination of strains are rare (Martinetti and Altwegg, 1990; Stanley *et al.*, 1992; Gruner *et al.*, 1994; Threlfall *et al.*, 1994). In a recent study (Morris *et al.*, 1992) it has been shown that in 1985 plasmid profiling was a reasonable means for typing *Salmonella* Enteritidis strains whereas in 1989 86% of sporadic case isolates examined had one single profile. This difference is consistent with

the observation that new clones have emerged in Europe (PT4) as well as in the United States (PT8) which are responsible for the majority of infections (Gruner *et al.*, 1994; Hickman-Brenner *et al.*, 1991).

PFGE of poultry isolates of *Salmonella* Enteritidis using three different restriction enzymes (*Xba*I, *Spe*I, *Avr*II) revealed two major patterns for each of the enzymes (Thong *et al.*, manuscript submitted). However, each pair of patterns was found in both PT4 and PT8 isolates. This indicates that phage types cannot be reliably separated by PFGE and that the two phage types might be closely related. In a study analysing exclusively PT4 strains (Powell *et al*,. 1994) 9 *Xba* I-profiles were found among 39 isolates. Most differences in the patterns found can be explained by single genetic events indicating a close relationship of the strains. In addition, 30/39 strains (including one isolated in 1967) belonged to one single PFGE-type. From these results it can be conclude that PFGE is neither a method that would allow separating strains of different phages types nor does it allow reliable discrimination within a given phage type.