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MOLECULAR EPIDEMIOLOGICAL TECHNIQUES FOR *SALMONELLA* STRAIN DISCRIMINATION

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1. ABSTRACT

*Salmonella*e are ubiquitous throughout nature where they infect or colonize humans, animals and even insects worldwide. The genus is comprised of a number of different antigenically distinct members, many of which have a particular niche within nature. Infection in animals and humans can be endemic or epidemic. Many nations have established extensive surveillance systems to track *Salmonella* infections and disrupt epidemic spread. Most of these surveillance projects rely on traditional serotype and phage type analyses to identify trends and potential outbreaks. Many clinical outbreaks cluster among a few serotypes so further discrimination is often needed. Molecular epidemiological techniques have been used to enhance surveillance and discriminate outbreak strains within these common serotypes. The institution of these techniques has led to enhanced detection of outbreaks worldwide. Molecular techniques used for *Salmonella* surveillance are described and comparisons of different molecular techniques are outlined. Overall, traditional serotype surveillance in association with one or several molecular typing techniques, especially chromosomal restriction fragment analysis with pulsed-field gel electrophoresis, appears to provide the most reproducible and comparable discrimination of epidemiologically-linked isolates at this time.

2. INTRODUCTION

*Salmonella*e are enteric gram negative organisms that are widely dispersed in nature. These organisms can reside as common commensals in the gastrointestinal tracts of animals and man or cause disease states that range from self-limited diarrhea to bacteremia with enteric fever or invasion of vascular structures, bone or other localized sites. Organisms can be highly host adapted, where they infect only a limited number of species, or can be much more ubiquitous. The most significant human host-adapted organism is *S. typhi*, the cause of typhoid fever. Man

remains the only known reservoir for these isolates. Similarly, *S. pullorum* and *S. gallinarum* are poultry-associated organisms that are so host-adapted that even upon transmission to man they usually remain nonpathogenic (1). More frequently, animal host-adapted organisms can be transmitted to man causing symptomatic disease. *S. choleraesuis* is normally a porcine organism though it can cause severe disease when transmitted to man. Other organisms, such as *S. typhimurium*, have a broad host range and these serotypes are responsible for the majority of human infections.

A number of studies have suggested that *Salmonella* infections tend to be most severe in patients with a weakened or underdeveloped immune system, in particular neonates, the elderly and those infected with the human immunodeficiency virus (2, 3). Infections occur endemically and epidemically throughout the world. The exact incidence of infection is unknown, though estimates suggest that over 1.4 million cases of Salmonellosis from nontyphoidal strains occur each year in the United States (U.S). Of these infections, 95% are thought to be associated with foodborne transmission (4). Most infections result from ingestion of contaminated, animal-derived food products such as meat and dairy products, though fruits and vegetables have been associated with transmission (5-9). Given the widespread distribution of food products today, some of these outbreaks have demonstrated remarkable geographic spread. Contaminated ice cream was thought to have infected up to 224,000 people from 41 states in the U.S. (10). Similarly, a contaminated snack food produced in Israel was responsible for spread of *S. agona* through the United Kingdom (U.K.), Israel and the U.S. (11). Much less common are cases of person-to-person transmission. Well-documented outbreaks in nurseries and other hospital units have been described (12, 13). However, foodborne transmission within the hospital needs to be distinguished from person to person spread. In 1987, a New York hospital experienced a large *Salmonella* outbreak where 42% of patients developed *S. enteritidis* infection. Epidemiological investigations identified infected eggs in a contaminated tuna-macaroni salad as the vehicle for infection (14). Finally, direct contact with colonized or infected animals, including exotic pets such as reptiles has been associated with human disease (15).

3. PHENOTYPIC TYPING TECHNIQUES

3.1. Serotype Analysis

Given the differing host ranges and disparities in clinical presentation, many attempts have been made to determine whether different subtypes of *Salmonella* might be responsible. As early as the 1920's, studies suggested that antigenic variation and serum agglutination reactions could be used to classify *Salmonella* isolates into various serogroups (16). Classification has been refined over the years and is generally attributed to the Kauffmann-White serotyping scheme based on the antigenic structure of the surface lipopolysaccharides (O antigens), flagellar proteins (H antigens) and capsular proteins (Vi antigens). Over 2400 serotypes have been characterized (17).

Simple serological analyses can rapidly classify most organisms into a distinct serotype (alternatively termed serovar). The fact that simple serological analysis can generate strain discrimination has led to a number of nation-wide surveillance programs that have been relatively successful, making nontyphoidal *Salmonella* spp. among the best tracked organisms. In 1962 the Centers for Disease Control and Prevention (CDC) instituted a National *Salmonella* Surveillance System database which gathered reports of laboratory confirmed cases from state public health laboratories around the U.S. (18). This *Salmonella* surveillance, supported primarily by serotype analysis, has identified important trends in the epidemiology of this pathogen. During the 1980's, a tremendous increase in *S. enteritidis* was identified, particularly in the Northeastern U.S. (19, 20). Studies linked *S. enteritidis* to contaminated shell eggs or foods that contained eggs (7, 21). During 1987-1997, five serotypes accounted for 66% of all clinical infections in which a *Salmonella* isolate was identified to the serotype level. *S. typhimurium* accounted for 24% of these isolates, *S. enteritidis* (22%), *S. heidelberg* (9%), *S. newport* (5%) and *S. hadar* (4%) followed (22). When clinical outbreaks were distinguished from sporadic infections, *S. enteritidis* was implicated in 55% of *Salmonella* cases associated with a clinical outbreak (22). Serological analysis usually remains the first step in an epidemiological investigation of *Salmonella* and may be sufficient for epidemiological investigations associated with uncommon serotypes (23). However, smaller labs often do not have access to the pools of serum required for this analysis and may need to rely on other techniques to analyze isolates.

3.2. Biotype analysis and phage typing

Since disease often clusters among a few serotypes, other methods have been used to further subdivide particular serotypes. Subtyping has been especially useful for the study of outbreaks among the most common serotypes such as *S. typhimurium* and *S. enteritidis*. A number of phenotypic properties have been used to differentiate isolates including biotype analysis of cellular metabolic enzymes, antimicrobial susceptibility testing and phage typing. Old et al. (24) used biotyping along with a number of other molecular typing techniques to determine that the rare *S. salinatis* was phylogenetically related to *S. sandiego*. In general, though, biotype analysis has been relatively insensitive for outbreak analysis

Individual isolates of many *Salmonella* serotypes vary in their susceptibility to lysis by different bacteriophages and this has led to a typing scheme based on reactivity to a panel of bacteriophage. Phage typing has led to the discrimination of over 200 *S. typhimurium* phage types (23) and, together with antimicrobial susceptibility analyses, led to detection of several large-scale, international epidemics including the dissemination of a multi-drug resistant clone of *S. typhimurium* DT104, (definitive phage type, DT, 104) (25, 26). Multidrug resistant *S. typhimurium* DT104 has been identified in cattle, poultry, swine, sheep and humans from the U.S. Western Europe, the United Arab Emirates, the Philippines and Israel (26). Given the relatively large number of phage

types in *S. typhimurium*, this technique remains one of the first subtyping methods in many countries (27).

Certain phage types, like serotypes, predominate in a region making this technique insensitive for outbreak analysis. *S. enteritidis* DT4 has become highly endemic in Britain, whereas DT8 has been more common in North America (28, 29). Additionally, phage susceptibility may be relatively plastic. Changes in phage type have been associated with the acquisition of a plasmid or changes in expression of lipopolysaccharide (30-33). Phage susceptibility changes can occur rapidly. Within six weeks, *S. enteritidis* isolated from one patient changed from DT4 to DT7 and DT9a (31). Phage typing is often an important early step in an investigation, but may need to be supplemented with other techniques for outbreak investigation involving a relatively common phage type. Furthermore, phage typing is technically demanding and requires the maintenance of stocks of biologically active phage and control strains; conditions that relegate this technique to reference laboratories (34).

3.3. Antibiotic Susceptibility Testing

Antibiotic resistance in many *Salmonella* serotypes is increasing with a number of organisms now showing multidrug resistance. Typhoid fever, unlike uncomplicated *Salmonella* gastroenteritis, requires appropriate antibiotic therapy. Epidemics of chloramphenicol resistant *S. typhi* were first recognized in the 1970's (35). Since this time, progressive antibiotic resistance has been noted worldwide with many isolates now showing resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline and trimethoprim (35). By 1995, 34% of *S. typhi* isolated in the U. K. demonstrated multidrug resistance (35). Most of the drug resistance has been plasmid-mediated though, recently, chromosomally-mediated fluoroquinolone resistance has become a real concern (36, 37).

Antimicrobial resistance has generated considerable attention in the nontyphoidal strains of *Salmonella* as well, particularly with the intense scrutiny being placed on the nontherapeutic use of antibiotics in food animal production. Multidrug resistance in bovine *S. typhimurium* isolates from the U.K. increased from 20% in 1981 to over 60% in 1990, while human isolates showed an increase from 5% to 19%. Early in this study the multidrug resistance was confined to isolates that belonged to DT 204c, but by 1990, multidrug resistance spread to new phage types (30). In contrast, a Danish study from 1997 found antibiotic resistance in human *S. typhimurium* isolates to be more common than among animal isolates. Further analysis revealed that many of the human isolates were acquired during foreign travel (38). Consistent with this finding, antibiotic resistance among *Salmonella* isolates is increasing in many countries including Taiwan, Thailand, Turkey and Kenya (39-42). In the U.S., the CDC, the U.S. Department of Agriculture and the U.S. Food and Drug Administration have an ongoing antimicrobial surveillance study (National Antimicrobial Resistance Monitoring System, NARMS), which has tracked resistance in *Salmonella* isolates from humans and food animals around the country since 1996 (www.cdc.gov/narms). Of over 1300 human isolates tested, 26% were resistant to one or more antimicrobial agents, 21% to two or more and 9% demonstrated resistance to at least ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (www.cdc.gov/narms).

Recognition of a new or unusual antibiotic resistance has often instigated epidemiological investigation. A rise in *S. typhimurium* DT104 cases was identified in Denmark during 1998. The isolates demonstrated the typical ampicillin, chloramphenicol, streptomycin, sulfonamide, tetracycline resistance known to be present in other countries, but the isolates also showed reduced susceptibility to quinolone antibiotics. Investigations linked the cases to pork consumption and were able to trace the infecting strain back to a particular swine herd (6). More recently, human and food animal *Salmonella* isolates have been identified that are resistance to extended spectrum cephalosporins as well as many other classes of antimicrobial agents (43-46). Molecular studies have revealed a common plasmid-mediated beta-lactamase gene responsible for the cephalosporin resistance and, in a single case, traced the infection from a patient back to an infected cow (43-46). In each of these studies, antimicrobial susceptibility testing highlighted a risk for an epidemic outbreak yet traditional phenotypic studies such as serotype and phage type analysis, often in conjunction with genotypic studies, were required to confirm and trace the origin of the infection. It should be noted that antimicrobial resistance genes are often located on mobile DNA elements, such as plasmids and transposons, which can be easily lost or acquired over short periods of time. Thus, antimicrobial resistance is not one of the most stable epidemiological markers for outbreak analysis.

4. GENOTYPIC TYPING TECHNIQUES

4.1. Plasmid Analysis

As methodologies and reagents for DNA extraction, restriction endonuclease digestion, hybridization and amplification have become standardized, genotypic methods for outbreak investigations and subtype discrimination have proliferated. Plasmid profile analysis was one of the earliest DNA-based subtyping schemes. *Salmonella* strains typically encode at least one plasmid with plasmid sizes ranging from 1 to 200 kilobases. Plasmid analysis has several limitations. Plasmids can rapidly be acquired or lost. Also, single predominant plasmids have become endemic within various serotypes. In sporadic isolates of *S. enteritidis* from Maryland, 88% of isolates contained a single 36-Mda plasmid (47). Similarly, only 1 of 56 *S. typhimurium* isolates failed to encode a 90 kb plasmid, which is thought to be a serotype specific virulence plasmid. Despite the ubiquitous nature of the 90 kb plasmid, profiling of the entire complement of plasmids in each strain was able to discriminate *S. typhimurium* strains isolated from a single poultry flock or closely related flocks (48). Plasmid analysis also was also able identify a multi-state outbreak of chloramphenicol resistant *S. newport* in humans that could be traced back to

contaminated beef and to dairy farms (49). In a testament to the power of combining a strong traditional epidemiological analysis with serological and genotypic tests, a peak of *S. muenchen* was noted in Ohio, Michigan, Georgia and Alabama. Epidemiological studies failed to identify a common food source responsible for this outbreak, but a strong correlation with marijuana use was identified. Marijuana obtained from affected households was contaminated with *S. muenchen* and the isolates from the different states showed a similar plasmid fingerprint suggesting interstate transfer of the contaminated drug (50). Plasmid profiling is most useful in an outbreak setting that is limited temporally and geographically (51, 52). Furthermore, this technique will only be successful if the serotype of interest carries multiple plasmids of differing sizes.

4.2. Chromosomal restriction fragment analysis and pulsed field gel electrophoresis (PFGE)

Analysis of chromosomal DNA, as opposed to mobile plasmid DNA, has become the standard for genotypic subtyping. Studies range from DNA restriction fragment analysis to hybridization and/or amplification techniques. Restriction digestion of chromosomal DNA has proven one of the most successful and reproducible techniques. Today most laboratories digest chromosomal DNA with a restriction endonuclease that recognizes relatively few sites within the chromosome, typically resulting in 10 to 30 independent DNA fragments that range in size from 10-800 kb. Special electrophoresis systems, which use pulsed currents that change polarity at defined intervals, are used to separate these large fragments. The process is often termed pulsed-field gel electrophoresis (PFGE). The choice of restriction endonuclease is somewhat empiric, but the most commonly used enzymes in *Salmonella* have been *Xba*I, *Spe*I and *Not*I. Comparisons of patterns from multiple enzymes can elucidate new subtypes and increase the discriminatory power of this technique (53). PFGE of 60 *S. enteritidis* isolates revealed 28 different *Xba*I restriction profiles and 26 with *Spe*I, yet when the patterns generated from both enzymes were combined, 32 different pulsed-field types could be identified (54). The Minnesota Department of Health used PFGE to determine whether molecular subtyping was able to detect unsuspected clusters or outbreaks of *S. typhimurium*. During a four-year period, 16% of isolates were linked to common source outbreaks. Of these, the authors felt that 62% of outbreak strains would have been missed without the use of PFGE molecular subtyping (55). PFGE has also been used to track outbreak strains occurring across national boundaries (56).

PFGE is characterized by a high degree of reproducibility both within and between laboratories (57-59). The recent introduction of computerized gel-based data collection and analysis systems allows better standardization between laboratories thus creating the ability to rapidly compare restriction fragment patterns from isolates analyzed from remote locations (57, 59). Large databanks that house PFGE patterns from isolates around the world will greatly enhance *Salmonella* outbreak detection. PulseNet, a molecular subtyping network for foodborne bacterial disease surveillance, has been active in developing standardized PFGE protocols and establishing a national database that is maintained at the CDC. An outbreak of *S. agona* linked to contaminated cereal was identified in 1998. PFGE, in association with PulseNet, was used to identify cases in adjoining states that were not initially thought to be at risk (57).

PFGE, however, is not always successful. Some serotypes, especially those with certain distinct phage types, can be so genetically homogeneous that multiple genotypic techniques fail to discriminate outbreak from non-outbreak strains. Ahmed et al. (60) evaluated PFGE to differentiate *S. enteritidis* DT8 strains that developed during a Canada-wide outbreak of gastroenteritis that was eventually traced to contaminated cheese. *Spe*I showed no advantage over *Xba*I and PFGE was not a reliable means of differentiating PT8 outbreak strains from sporadic PT8 isolates. Successful discrimination was only achieved with a combination of intensive epidemiological, genotypic and phenotypic methods (60). Additionally, certain serotypes may be more susceptible to genetic rearrangements that can alter the PFGE pattern, even within an outbreak (61). Three outbreaks of *S. typhi* demonstrated two different PFGE patterns during each outbreak. Chromosomal analysis demonstrated genetic reassortment events that led to the variation in PFGE patterns within particular outbreaks (62). PFGE, too, is relatively slow, often taking three days to complete, and equipment costs can approach $20,000 for the gel system alone with costs increasing significantly when computer imaging and data analysis systems are included.

4.3. Chromosomal Restriction-hybridization techniques

4.3.1. Ribotype analysis

Fingerprinting of rRNA coding sequences, termed ribotyping, adds a second step to chromosomal restriction fragment analysis though it simplifies the banding pattern. Multiple copies of the rRNA operon are present within the *Salmonella* chromosome (52). The rRNA genes themselves are quite homologous among these copies and between isolates, but the intervening sequences vary in length and nucleotide composition. Ribotyping begins with separating endonuclease-digested chromosomal DNA on agarose gels, DNA then is transferred to a membrane and fragments are hybridized to a probe that recognizes 16S and 23S rRNA. Analysis of multiple restriction endonucleases can improve the discriminatory powers of ribotyping (48).

Ribotype analysis is clearly able to subtype some of the isolates that fall within some common serotypes and phage types (63, 64). Lin et al. (65) detected 7 different ribotypes among 17 *S. enteritidis* PT 8 isolates when chromosomal DNA was digested with *Sph*I. However, studies have identified isolates that belong to different phage types yet demonstrate identical ribotypes (52, 53, 66). Comparisons of ribotyping with PFGE have been somewhat unpredictable and often depend on the enzymes used for digestion as well as the nature of the population being tested. Several studies have found PFGE to be more

discriminating than ribotype analysis (54, 66, 67) while others have found the two procedures equivalent (68) or ribotype analysis superior (69). Ribotype analysis using two restriction enzymes, *Pst* I -*Sph*I or *Hind*III - *Eco*RV, can improve discrimination (52, 69, 70). As mentioned above, particular care must be taken when analyzing chromosomal patterns of *S. typhi*. The rapid genomic reassortment that occurs in *S. typhi* can affect ribotype analysis (71).

Though most laboratories continue to perform ribotyping manually, machinery has been developed to perform this entire procedure in an automated fashion. Data is stored electronically and the banding pattern from a particular organism can be compared to the entire databank stored in the computer. In contrast to PFGE, the time required to perform automated ribotyping is minimal; hybridization results can be obtained within 4 hours. A recent study tracking the rise of a multi-drug resistant, cephalosporin-resistant *S. newport* proposes to use automated ribotyping as a way to rapidly identify the newport serotype and PFGE to further evaluate strain associations (66). The major drawbacks of automated ribotyping are the high reagent costs per isolate and the cost of the automated riboprinter itself.

4.3.2. *IS*200 Analysis

A second chromosomal DNA digestion-hybridization scheme, called *IS*200 typing, has been used to evaluate the molecular relationships between *Salmonella* isolates. *IS*200 is a 708 bp insertion sequence that is present in multiple copies within the *Salmonella* chromosome (72). Hybridization of digested chromosomal DNA with an *IS*200 probe has been useful in describing the clonal heritage of *Salmonella* from various serotypes, but has not been as discriminating as phage typing itself for *S. enteritidis*, *S. typhi* and others (73). For certain phage types of *S. typhimurium*, such as the multidrug resistant DT204c and 193 types common in the U.K., *IS*200 typing can result in strain discrimination and in some studies has been superior to PFGE and ribotyping (27, 74, 75). More frequently, PFGE has performed better than *IS*200 typing (53, 68, 76, 77).

4.4. Locus-Specific PCR

4.4.1. PCR based *IS* 200 and Ribotype Analysis

Polymerase chain reaction (PCR)-based studies have been introduced that closely mirror techniques described above. Milleman et al. (78) describe an *IS*200 PCR that proved slightly less discriminating than a PCR-based ribotyping technique for *S. typhimurium*. Outward facing primers complementary to the ends of *IS*200 were used in this study to identify the number of copies of *IS*200 and the relative insertion positions within the genome. PCR ribotyping amplifies the 16S-23S rRNA intergenic spacer regions within the chromosome (79, 80). PCR ribotyping has been suggested as a rapid primary tool in outbreak investigation since the technique typically reflects serotype discrimination. In some serotypes, *S. derby*, *S. infantis* and *S. typhimurium*, the technique generates some additional discrimination (79). The utility of these studies will be better understood when they are applied in outbreak or other large field investigations

4.4.2. Integron Analysis

PCR based on other known loci in the chromosome have been useful in certain outbreaks. Class I integrons are mobile DNA elements that often encode one or more antimicrobial resistance genes (81, 82). Primers have been designed that amplify the variable cassette region where these genes are inserted (83). A highly multidrug resistant *S. typhimurium* DT104 clone containing a very conserved antibiotic resistance cluster encoded within integrons has emerged worldwide (25, 84-87). Integron PCR has been used to help identify DT104 clones carrying the conserved 13,000 bp fragment that encodes two integrons containing ampicillin, chloramphenicol, streptomycin, sulfasoxazole and tetracycline resistance genes (88). Integron PCR has also been used to complement other typing measure in studies of *S. virchow* though, in this case, the variability (as opposed to the similarity) of the inserted integron cassettes was used to differentiate strains (70). These locus-specific PCR techniques show good reproducibility within and between laboratories (58), but their use is limited to a few known loci. Also, care must be taken with integron analyses performed over time since these are mobile DNA elements.

4.5. Arbitrarily Primed PCR (AP PCR)

Arbitrarily primed PCR (AP PCR) is a DNA fingerprint technique that uses short (typically 9-15 bp) random sequence primers that hybridize at multiple random chromosomal sites under low stringency conditions. If one copy of the primer can bind on the plus-sense strand and a second copy hybridizes to a site on the minus-sense strand of DNA, and the sites are within 1-2 kb, a DNA fragment will be amplified. All bacterial isolates should be typeable using this technique and the material costs and labor are low. However, identification of appropriate primers is arbitrary and can require significant test development time. Lin et al. (65) screened 65 primers before selecting six primers that resulted in multiple AP PCR banding patterns from *S. enteritidis* isolates. After this laborious selection process, AP PCR did perform better than phage typing, ribotyping and PFGE. This technique has successfully differentiated isolates from identical phage types (65, 89), while in certain serotypes, such as *S. dublin*, ribotyping or other techniques have been superior (90). AP PCR, is rapid and relatively inexpensive, but has several drawbacks. Banding patterns can vary with pH, magnesium and DNA concentrations and even the source of DNA polymerase (51, 65, 91). The banding patterns, too, are typically comprised of several dominant and several less intense bands. The intensity of these faint bands can vary from gel to gel and complicate computer-based gel analysis (92). This technique has poor interlaboratory reproducibility and is best used to evaluate a set of isolates analyzed in a single amplification reaction separated on a single gel (51).

4.6. Repetitive Sequence PCR (REP PCR)

Interspersed, untranslated, repetitive DNA sequences have been identified in many bacterial genomes

though the exact function of these structures is unclear (93-95). The repetitive extragenic palindromic (REP) element, Enterobacterial repetitive intergenic consensus (ERIC) sequence and BOX A elements have been identified in *Salmonella*e. REP PCR (which often refers to PCR using primers to any of the three repetitive elements) has been shown to discriminate isolates to the serotype level (96, 97). Johnson et al. used REP PCR with ERIC2, BOXA1R or a composite of the two sets of primers to analyze a hospital cafeteria associated outbreak of gastroenteritis caused by *S. infantis.* This technique was quite effective at serotype discrimination and even identified several historical serotyping errors within their collection of isolates. However there was no evidence for subserotype discrimination and the technique could not distinguish outbreak strains from unrelated *S. infantis* controls (96, 97). Not all serotypes appear to be separable by REP PCR. Milleman et al. (98) discovered a common ERIC PCR pattern shared by *S. typhimurium* and *S. enteritidis* strains. In another study ERIC PCR demonstrated a unique fingerprint for almost every isolate, but did not identify serotypes (92). Intralaboratory variability also has been problematic (59, 92, 97). An increased annealing temperature (96, 99) and the use of duplicate fingerprints for each isolate with a third PCR analysis for discrepant fingerprints (96) can improve reproducibility. Also, REP and ERIC primers may not agree in their subtyping schemes. REP PCR appears to be a screening tool or surrogate serotyping method best used to analyze a specific outbreak setting since ribotype or PFGE analyses often are better able to discriminate isolates to a subserotype level (96-98). Additionally, within a single laboratory, AP PCR profiles often differentiate among serotypes better than REP or ERIC PCR (70, 98). As with many of the techniques discussed, combinations of these procedures with or without traditional phenotypic methods are frequently superior to the use of a single technique (70, 90, 98, 100).

4.7. Amplified fragment length polymorphism (AFLP)

Many variations on PCR-based DNA fingerprinting techniques are being developed to improve the reproducibility and speed of typing. Amplified fragment length polymorphism (AFLP), also termed infrequent restriction site PCR (IRS PCR) starts with digestion of DNA with two restriction endonucleases, the ends are linked to oligonucleotide adapters and PCR is performed using adapter-specific primers (101). Optimization of restriction enzymes and adapter-specific primers is ongoing for the *Salmonella*e (59, 102), but the technique appears more reproducible than REP and AP PCR techniques (59, 101). Some of the studies have shown specificity to the serotype level with occasional subserotype discrimination (59, 103). Several groups have employed a fluorescent amplified fragment length polymorphisms (FAFLP) technique that followed the same principles of AFLP yet the adapter-specific primers were tagged with a fluorescent moiety (104, 105). Fluorescent-tagged fragments are then accurately sized on an automated sequencer. FAFLP analysis of *S. typhimurium* generated 45-50 fragments ranging in size from 80-430 bp, though only a subset of these fragments were polymorphic among the strains. FAFLP grouped the isolates into four distinct clusters while PFGE generated three clusters. Sizing was enhanced by incorporation of a fluorescent internal marker (104). This accurate sizing, combined with the ability to acquire and analyze the data as a gel image, electrophorogram or in a tabular data format will allow comparison of patterns among different laboratories or within databanks (101). At this time, FAFLP appears quite promising, though further studies comparing this technique to other molecular and phenotypic typing are needed. Disadvantages include the need for a greater technical expertise than required for REP or AP PCR techniques and set up costs may be prohibitive until automated sequencers become more affordable.

4.8. Multilocus Sequence Typing (MLST)

DNA sequence analysis has been used to characterize the genetic relationships and phylogeny of a number of bacterial pathogens including *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Neisseria meningitidis*. Multilocus sequence typing (MLST) is a molecular typing strategy that compares DNA sequences from portions of housekeeping or virulence genes and/or rRNA sequences (106). Over 230 Salmonella isolates were recently characterized by MLST based on sequences from the 16S RNA, *pduF*, *glnA* and *manB* genes(107). These results were compared to PFGE and serotype analysis. MLST was able to differentiate strains better than PFGE, though not all genes performed equally. Among the four loci, only *manB* demonstrated clusters among the clinical and environmental strains. As expected, the 16S rRNA locus showed significant homogeneity among the isolates and grouped most isolates together. MLST shows great promise for accurate strain discrimination with data that can be accurately shared between laboratories. However, like FAFLP, the universal appeal of this technique will be improved when automated sequence machinery becomes more affordable and labs can develop familiarity with complicated DNA sequence analysis and statistical software

5. PERSPECTIVES

Overall the *Salmonella*e demonstrate significant phenotypic diversity. Several phenotypic typing techniques have been developed that have been used successfully for decades. Over the years, serotype and phage type analyses have been particularly useful as evidenced by the success of the National *Salmonella* Surveillance System, that has been ongoing since 1962, and many other national surveillance projects throughout the world. However, these techniques have often been relegated to reference laboratories making rapid analysis by an individual laboratory difficult. It is clear, too, that the majority of human cases cluster among a few serotypes making subserotypic discrimination crucial in epidemiological investigations. A number of genotypic techniques have been analyzed and many are able to rapidly and easily identify certain organisms to a level commensurate with serotyping. Unfortunately, though, no one technique is optimal for analysis of all *Salmonella* serotypes and the best discrimination has resulted from

combinations of techniques, often a combination of phenotypic and genotypic techniques. At this time, major reference institutions rely on serotype analysis followed by PFGE as the gold standard for strain discrimination, particularly since computer-based gel analysis techniques have allowed the comparison of PFGE patterns from multiple laboratories. PCR-based techniques, though, are more rapid and within a particular laboratory can be used as a primary screening tool for strain discrimination. Better standardization between laboratories will be required before any of the PCR techniques can become the method of choice. Additionally, validation in outbreak situations involving varied serotypes will be required to prove these techniques effective in the field. Strain typing using any method is most effective when typing is integrated with data from a thorough epidemiological investigation (51). Therefore, recognition of an unusual pattern of infection should trigger both a traditional epidemiological approach complemented by laboratory investigations.

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