Using the Tools of Molecular Biology to Aid in Foodborne Disease Investigations

Leslie A. Wolf, PhD
North Carolina State Laboratory of Public Health
Raleigh, NC

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Overview of Presentation

- Introduce key molecular biology terms
- Discuss a variety molecular subtyping techniques
- Explain PulseNet
- Highlight a number of foodborne disease outbreak investigations that used molecular subtyping for epidemiologic purposes

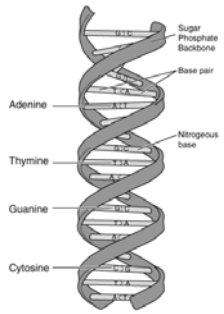
General Molecular Biology Terms

- Genome
- DNA
- RNA
- Gene
- PCR
- Primers
- Restriction Enzyme
- Fingerprinting
- Mutation

Genome

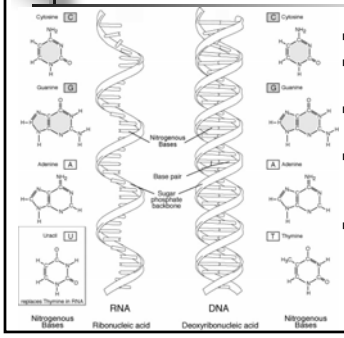
- The entire nucleic acid molecule of an organism that encodes enzymes, proteins, and other structural components
- For most bacteria, a single circular molecule containing DNA

DNA



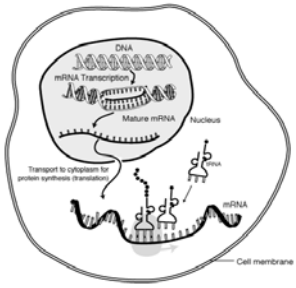
- Deoxyribonucleic acid
- Sugar-phosphate backbone and nitrogenous bases form the helical double-stranded molecule
- Adenosine pairs with Thymidine
- Guanosine pairs with Cytosine

RNA

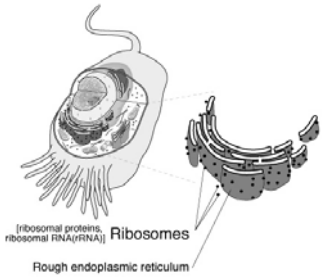


- Ribonucleic acid
- Contains Uracil instead of Thymidine
- Messenger RNA (mRNA) encodes proteins
- Transfer RNA (tRNA) chooses correct amino acid to build proteins
- Ribosomal RNA (rRNA) provides docking structure for protein assembly

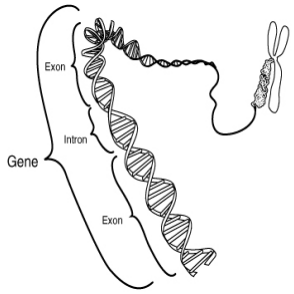
mRNA/tRNA



Ribosomal RNA



Gene

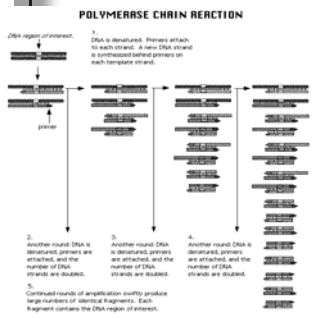


- A portion of the genome that encodes a specific product, such as a protein or enzyme or other macromolecule
- Example: the gene *stx*1 encodes a shiga toxin from *E. coli* O157:H7

Primers

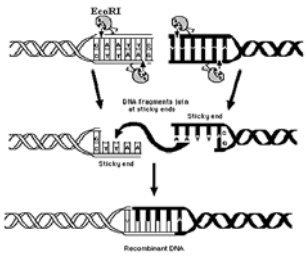
- Short pieces of DNA that bind to sequences of denatured DNA
- Sometimes designed to be highly specific to target a certain gene
- Sometimes designed to be degenerate to increase chances for binding

PCR



- Polymerase Chain Reaction
- Method developed by K. Mullis as a way to copy DNA *in vitro*
- One copy of a gene can be amplified 10^6 times so that it becomes "visible" by gel electrophoresis or by fluorescent assays

Restriction Enzyme



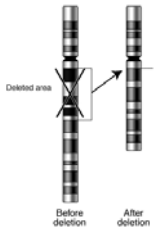
Restriction Enzyme Action of EcoRI

- Enzymes produced naturally by many strains of bacteria as a defense mechanism
- Enzymes cut DNA at specific combinations of A, G, C and T.
- Named systematically: EcoR1 is the first enzyme isolated from *Escherichia coli*

Fingerprinting

- A term used to describe any method that provides additional information at the molecular level to distinguish among bacterial strains

Mutation

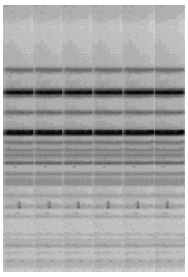


- A change in the nucleic acid sequence that may or may not be readily observed

(lethal ⟺ neutral)

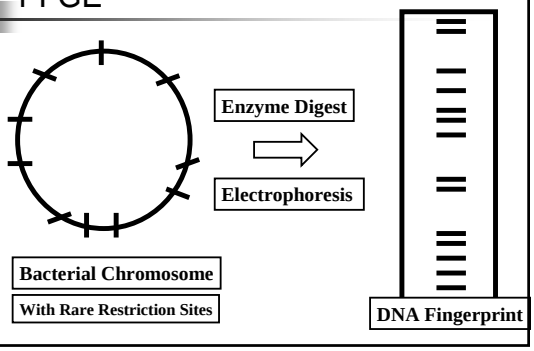
- Insertion of A, T, G or C
- Deletion of A, T, G or C
- Substitution of bases (point mutations)
- Inversions

Pulsed-Field Gel Electrophoresis



- PFGE is gold standard for bacterial subtyping
- Looks at whole genome of bacterial pathogens using rare cutting restriction enzymes
- Fragments are 30 kb to several hundred kb in size
- Labor-intensive, but readily standardized

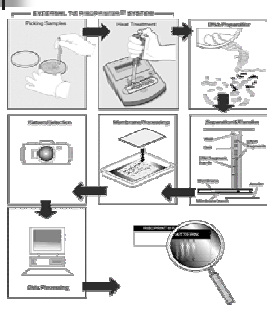
PFGE



Restriction Fragment Length Polymorphism

- RFLP is a method of detecting changes in restriction sites at specific genetic loci
- Fragments are generally less than 1 kb
- Useful only for genes that are variable

Ribotyping



- Automated system available from Dupont-Qualicon **(www.qualicon.com)**
- Variation of RFLP, using primers to rRNA gene targets
- Chemiluminscent detection of banding patterns due to probe binding sites

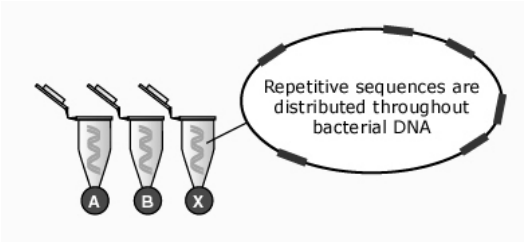
Randomly Amplified Polymorphic DNA

- RAPD uses short random sequence primers, 9-10 bases long
- Amplifies chromosomal DNA at low annealing temperatures
- Number and location of random primer sites vary for different strains

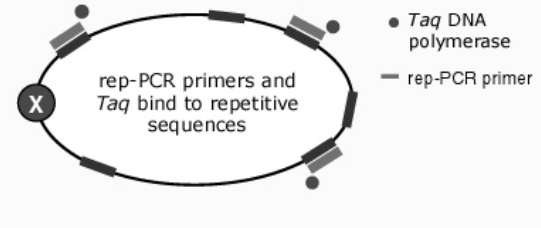
Repetitive Extragenic Palindromic Element PCR

- PCR based amplification of REP or ERIC sequences
- Short amplicons, 38 or 126 bp in length
- Highly conserved and widely dispersed in enteric bacterial genomes (J. Versalovic
- *et. al.*, 1991, *Nucleic Acids Research* and www.bacbarcodes.com)

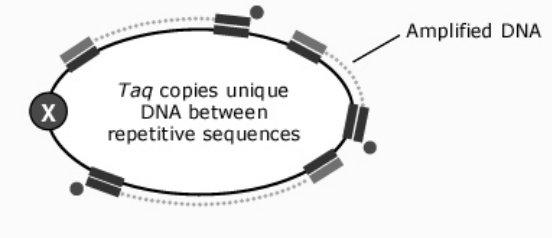
Rep-PCR



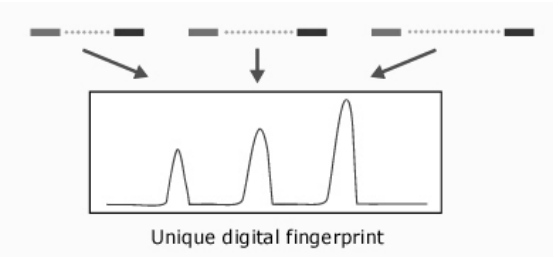
Rep-PCR



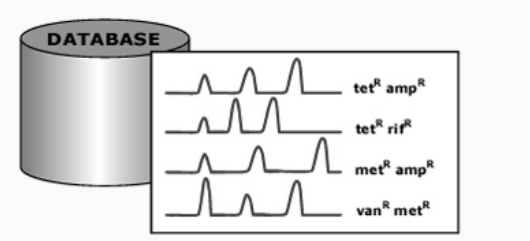
Rep-PCR



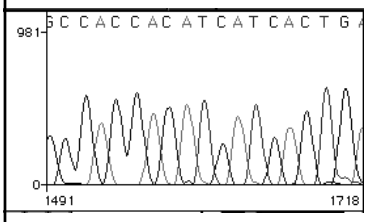
Rep-PCR



Rep-PCR



Next Generation Subtyping Methods



- DNA Sequencing
- MLST
- VNTR
- MLVA
- PATS
- Microarray

Multilocus Sequence Typing

- PCR amplification of several housekeeping genes
- PCR amplification of one or more variable genes
- DNA sequencing of amplification products

PulseNet

The National Molecular Subtyping Network for Foodborne Disease Surveillance

PulseNet

- Initiated in 1998 in response to a large outbreak of *E. coli* 0157:H7 in western US
- Standardized PFGE procedures allow participating laboratories to rapidly compare results electronically
- Certification and proficiency testing are required of all participating laboratories

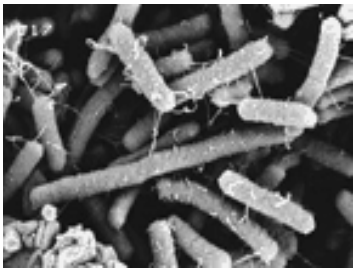
PulseNet

- Participants include CDC, state public health laboratories, NYC, Houston, and LA city laboratories, FDA and USDA laboratories
- Seven area laboratories provide additional capacity (WA, UT, TX, MN, MI, VA, MA)
- National databases for *E. coli* O157:H7, *Salmonella, Listeria monocytogenes* and *Shigella sonnei* are managed by CDC

PulseNet

- Goals of national surveillance system are to identify clusters of foodborne disease and to assist in epidemiologic investigations
- Ideally this will limit foodborne outbreaks by identifying sources of contamination and by raising awareness of food safety issues

Escherichia coli O157:H7

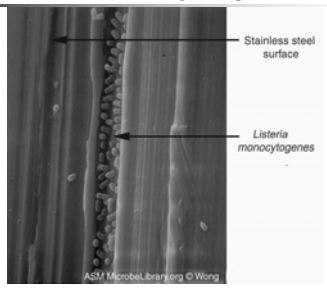


SEM of *Escherichia coli* ©Scott Kachlany, author. Licensed for Use, ASM MicrobeLibrary

E. coli O157:H7

- Gram-negative rod, producing shiga toxins 1 or 2, low infectious dose
- Causes nausea, abdominal cramping and frequently, bloody diarrhea
- Elderly and young children most at risk of severe illness; HUS is a serious complication in young children
- A number of food vehicles identified during outbreaks, including hamburger, lettuce, and unpasteurized apple juice

Listeria monocytogenes



SEM of *Listeria monocytogenes* ©Amy C. Lee Wong, author. Licensed for Use, ASM MicrobeLibrary

Listeria monocytogenes

- Gram positive rod, intracellular pathogen
- Causes sepsis or meningitis in immuno-compromised hosts; causes a mild, flu-like illness in pregnant women; febrile gastroenteritis in immunocompetent hosts
- Causes spontaneous abortions, premature births, and newborns with bacteremia, leading to fetal deaths
- Soft cheeses, unpasteurized milk products, and ready to eat deli meats are common vehicles of transmission

Salmonella enterica

- Gram negative rod, 2500 serotypes
- Sudden onset of illness, causing fever, nausea, abdominal pain and diarrhea for 4-7 days
- Second most common cause of bacterial gastroenteritis in US, usually in infants and young adults
- A number of food vehicles have been implicated in outbreaks, including toasted oat cereal, cantaloupe, mangoes, and eggs

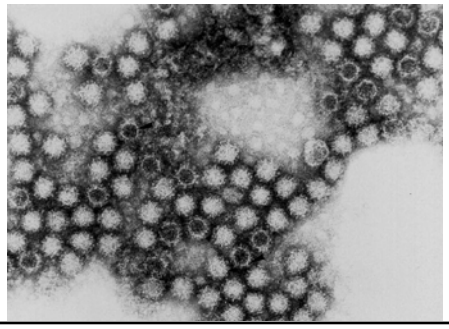
Campylobacter jejuni

- Microaerophilic gram negative,"seagull" shaped bacterium
- Prodrome of 1-2 days with fever, headache, muscle pain and malaise with acute onset of diarrhea, cramping, abdominal pain, fever
- High percentage of raw poultry contaminated with *C. jejuni;* raw milk may be contaminated
- Most common cause of bacterial gastroenteritis in US, usually infants and young adults; mostly sporadic cases, few outbreaks

Shigella sonnei

- Gram negative rod, low infectious dose
- Most common in daycare settings, no animal reservoir
- Causes acute onset of watery or bloody diarrhea, nausea, abdominal pain, fever, malaise lasting 4-7 days
- Some foodborne outbreaks have occurred in recent years, including parsley and lettuce

Calicivirus



Calicivirus/Norwalk-like Viruses

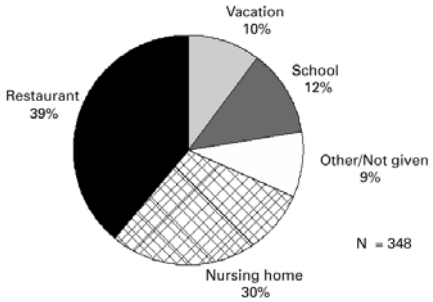
- Non-cultivable RNA viruses, only detected by electron microscopy and reverse-transcriptase PCR
- Many foodborne outbreaks, particularly in oysters contaminated with raw sewage, and via restaurants and catering establishments
- Nausea, vomiting, abdominal cramps and diarrhea for 24-48 hours; headache and low grade fever may also occur

MMWR 50(RR09): 1-18, 2001

TABLE 1. Characteristics of "Norwalk-like viruses" that facilitate their spread during epidemics

Characteristic	Observation	Consequences
Low infectious dose	<10² viral particles	Permits droplet or person-to-person spread, secondary spread, or spread by fecal-borne
Prolonged asymptomatic shedding	≤2 weeks	Increased risk for secondary spread or problems with control regarding food handlers
Environmental stability	Survives ≤10 ppm chlorine, freezing, and heating to 60°C	Difficult to eliminate from contaminated water; virus maintained in ice and steamed oysters
Substantial strain diversity	Multiple genetic and antigenic types	Requires complex diagnostics; repeat infections by multiple antigenic types; easy to underestimate prevalence
Lack of lasting immunity	Disease can occur with reinfection	Childhood infection does not protect from disease in adulthood; difficult to develop vaccine with lifelong protection

FIGURE 2. Mode of transmission of 348 outbreaks of gastroenteritis reported to CDC during January 1996–November 2000*



*Source: Fankhauser RL, Noel JS, Monroe SS, Ando T, Glass RI. Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. J Infect Dis 1998;178:1571–8; and CDC, unpublished data, 1997–2000.

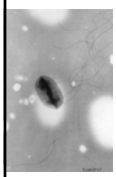
E. coli O157:H7 outbreak, Robeson County, NC 2001

- November 28th, physician reports to RCHD that several children had symptoms of bloody diarrhea
- Culture confirmed 11 children had O157:H7
- PFGE analysis of first several isolates showed indistinguishable patterns with two enzymes
- Between mid-November and late December, 203 suspect cases of *E. coli* O157:H7 infection were identified

E. coli O157:H7 outbreak

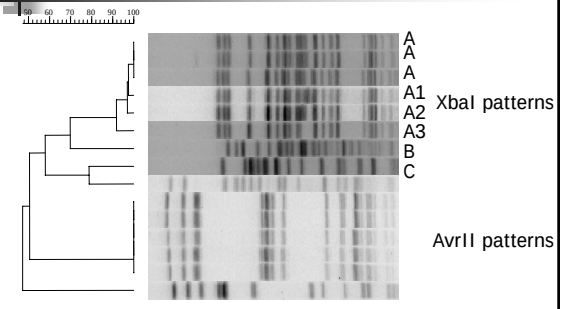
- Two events held at local schools: food tasting for Native American Cultural Heritage month and tasting of homemade butter
- After interviewing confirmed cases and controls, three suspect foods emerged: hamburger, string beans and butter

E. coli O157:H7 outbreak



- After controlling for exposure to hamburger and string beans, a highly significant association between illness and eating butter made with unpasteurized milk remained
- Manure from milk cows tested positive for shiga toxin by EIA

E. coli O157:H7 PFGE Patterns



Recommendations

- Educate school age children about proper handwashing techniques
- Examine school exclusion policies for illness in different age groups
- Discourage production and distribution of home-made food made from raw milk products

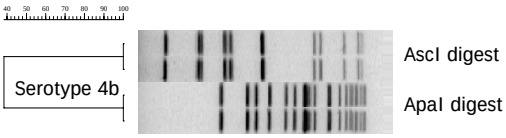
L. monocytogenes outbreak, Forsyth County,NC 2000

- 12 cases in hispanic population between October 2000 and January 2001
- 11 were females, 10 were pregnant
- Infections with *L. monocytogenes* led to 5 stillbirths, 3 premature deliveries and 2 infected newborns
- Illness associated with eating cheese purchased from door-to-door vendors

L. monocytogenes outbreak

- Investigation of these cases revealed that Mexican-style soft cheese was made in private homes from raw milk
- Home-made cheese sold door-to-door or in parking lots
- Found unlabeled home-made cheese in local Latino grocery stores

L. monocytogenes PFGE Patterns

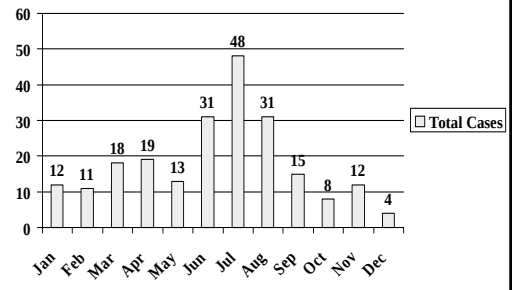


Ribotyping patterns were indistinguishable: DUP1042

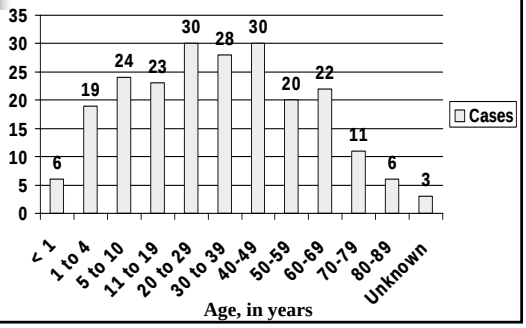
Recommendations

- Limit sale of raw milk products to regulated processors
- Educate hispanic communities about the dangers of eating unpasteurized cheeses while pregnant
- Make listeriosis a reportable illness in North Carolina
- *MMWR* 50(26): 560-562, July 6, 2001

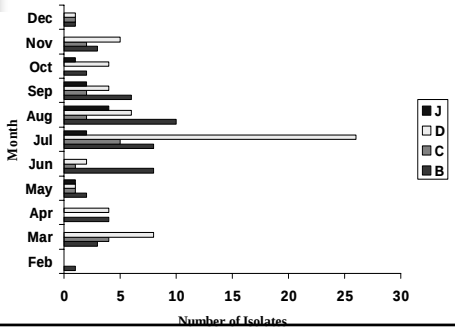
North Carolina *Salmonella* Enteritidis Case Numbers by Month, 2001



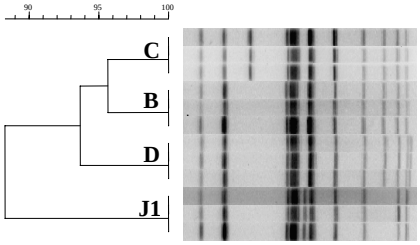
Age Distribution of *S.* Enteritidis cases in North Carolina, 2001



Number of Isolates with Common *S.* Enteritidis PFGE Patterns, 2001



Common *S*. Enteritidis PFGE Patterns, North Carolina 2001



Conclusions

- PFGE subtyping of *S*. Enteritidis isolates during an outbreak is useful for separating outbreak from non-outbreak cases
- Additional subtyping of *S*. Enteritidis, such as phage typing, may be required
- Safe handling of eggs, particularly at the point of consumption, is necessary to prevent infection with *S*. Enteritidis

Norwalk-like virus outbreak, Texas 1998

- March 1998, 23 students from local university treated in ER for acute gastroenteritis
- Investigation showed that illness was associated with eating at the campus cafeteria or deli bar during lunch/dinner
- Food handler who prepared deli ham and sandwiches wore gloves, but took care of infant with watery diarrhea two days before preparing food

NLV outbreak, Texas

- 50% of stools submitted by students demonstrated evidence of NLV by reverse transcriptase PCR
- The only food that tested positive for NLV by RT-PCR was deli ham
- Sequence analysis of NLV from ill students, deli ham, and the infant of food handler had identical sequences in the capsid gene
- *Journal of Infectious Diseases*, 2000, 181:1467-1470

Recommendations

- Closed deli bar once became suspected as source of outbreak
- Consider paid leave for food handlers with gastroenteritis
- Further studies needed to determine duration of shedding infectious NLV
- Educate food handlers regarding personal hygiene when caring for family members (especially diapered infants) with gastroenteritis

Questions?