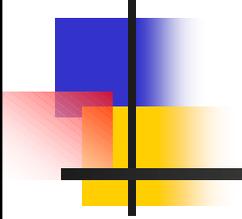


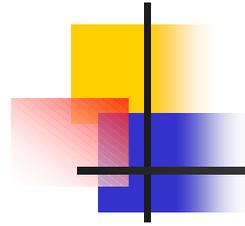
Using the Tools of Molecular Biology to Aid in Foodborne Disease Investigations



Leslie A. Wolf, PhD

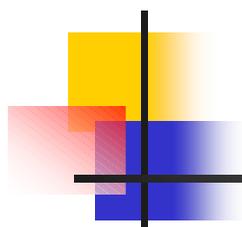
North Carolina State Laboratory of
Public Health
Raleigh, NC

May 14, 2002



Overview of Presentation

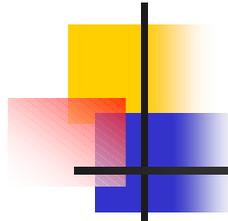
- Introduce key molecular biology terms
- Discuss a variety molecular subtyping techniques
- Explain PulseNet
- Highlight a number of foodborne disease outbreak investigations that used molecular subtyping for epidemiologic purposes



General Molecular Biology Terms

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

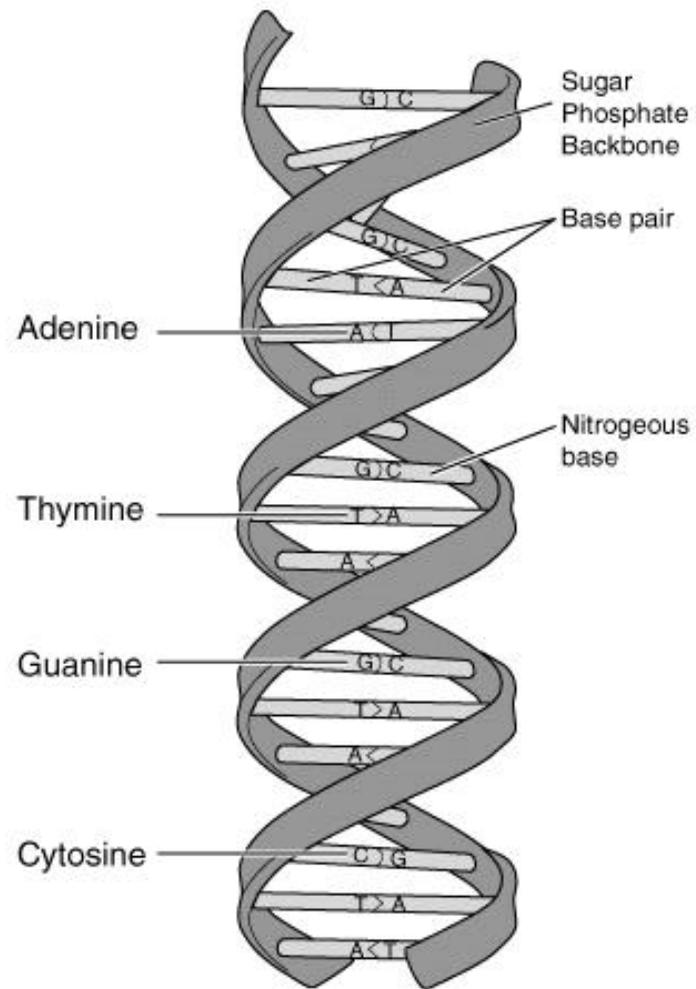




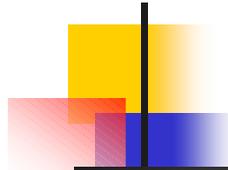
Genome

- 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

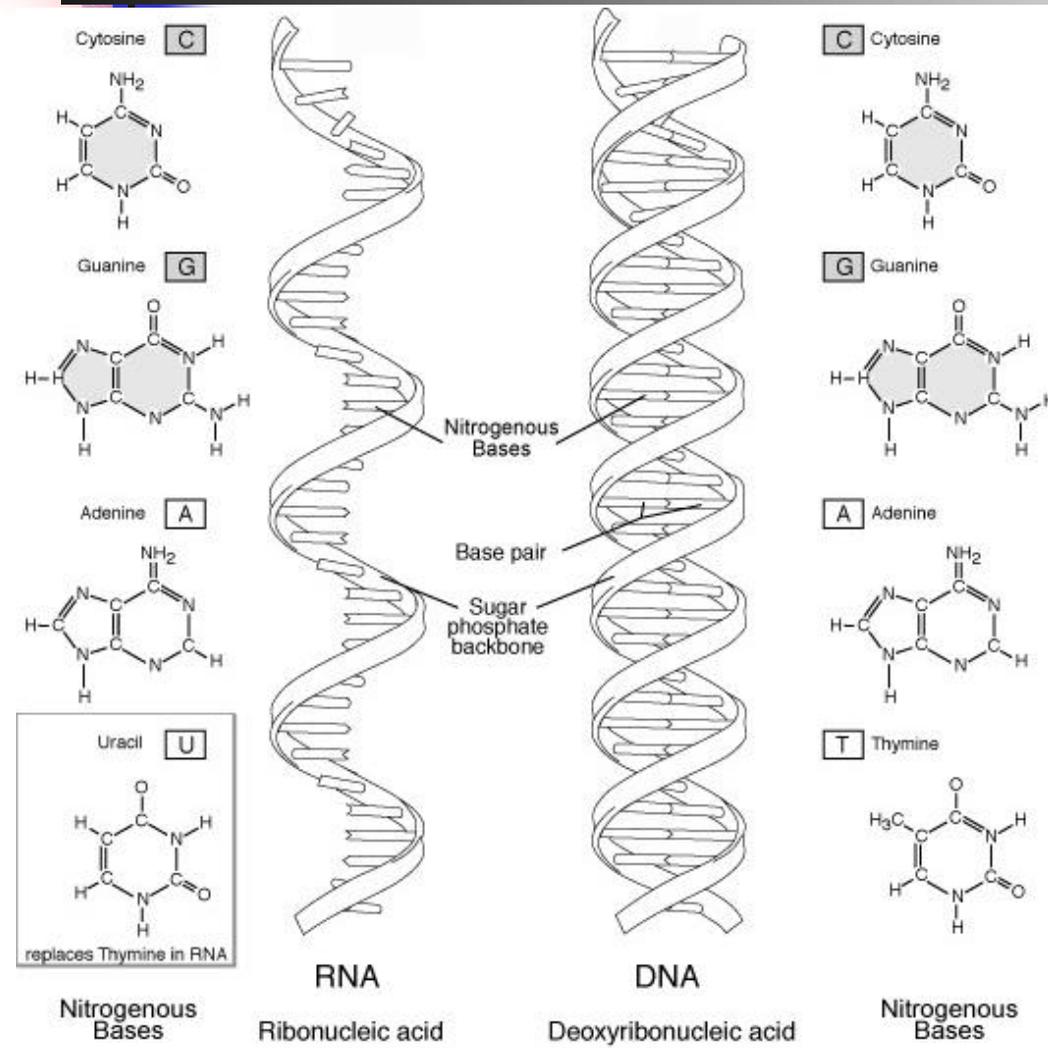
DNA



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

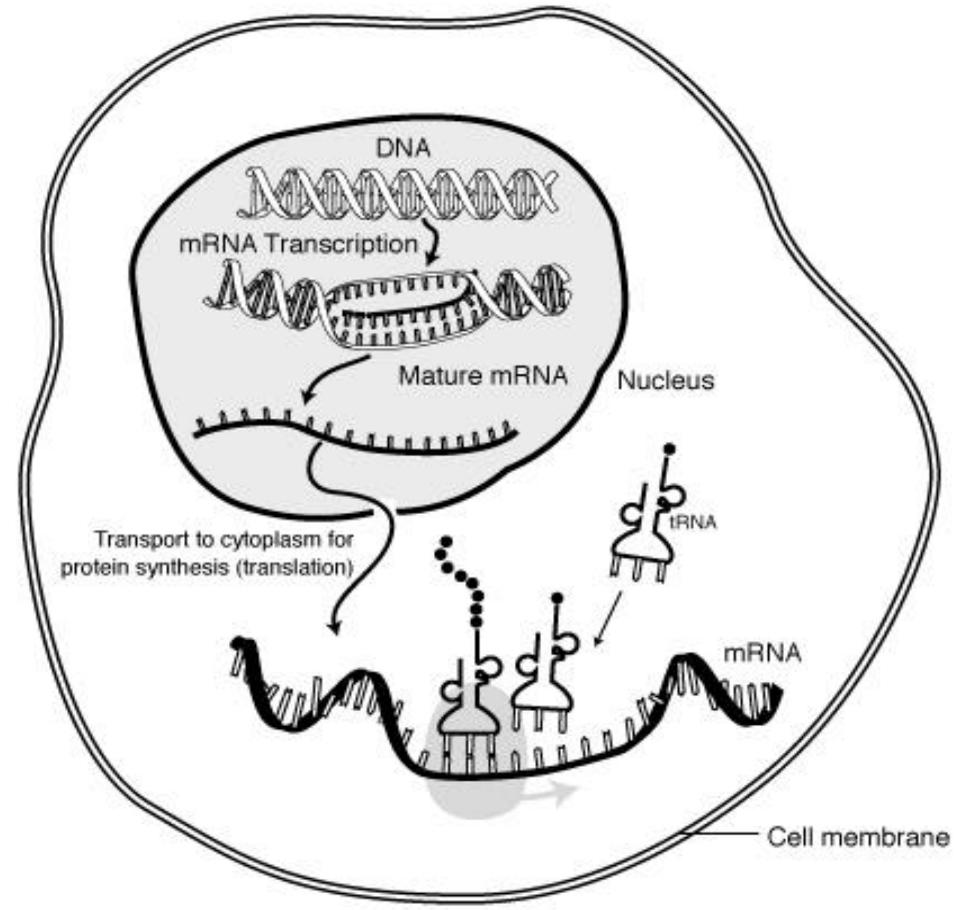


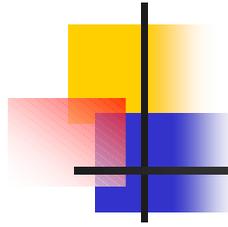
RNA



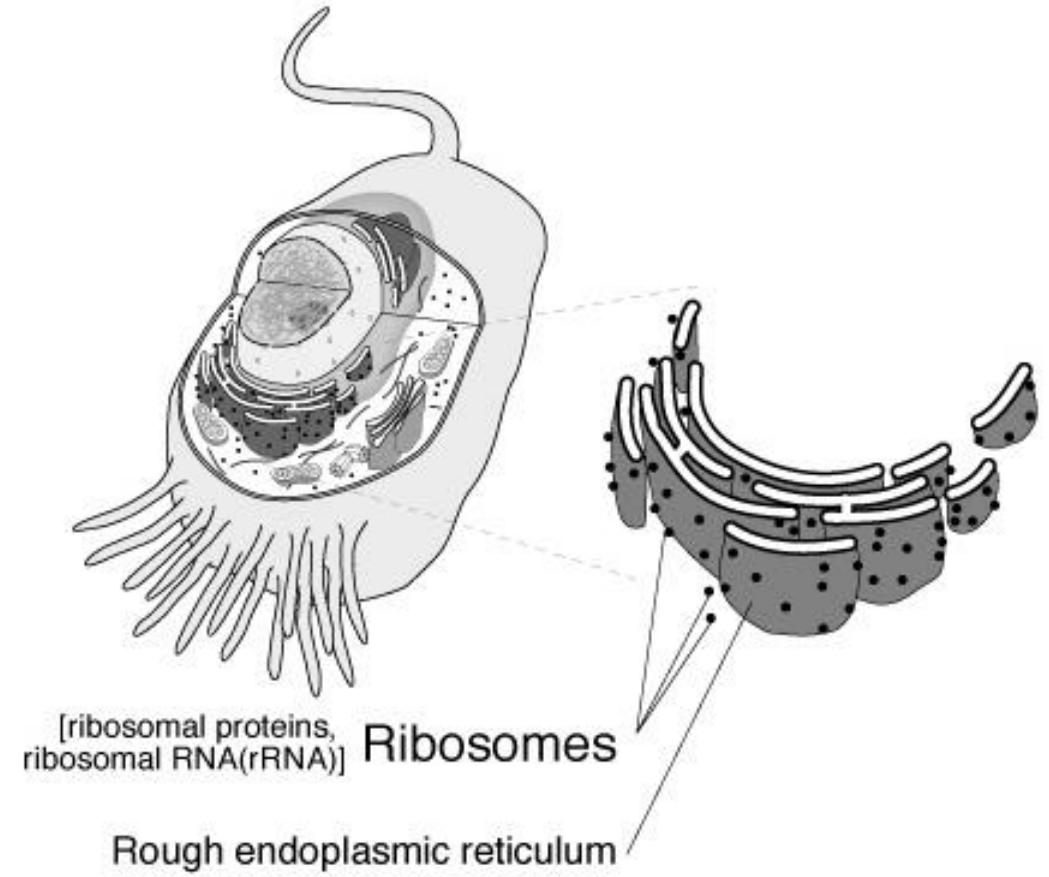
- Ribonucleic acid
- Contains Uracil instead of Thymidine
- Messenger RNA (mRNA) encodes proteins
- Transfer RNA (tRNA) chooses correct amino acid to build proteins
- Ribosomal RNA (rRNA) provides docking structure for protein assembly

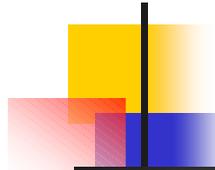
mRNA/tRNA



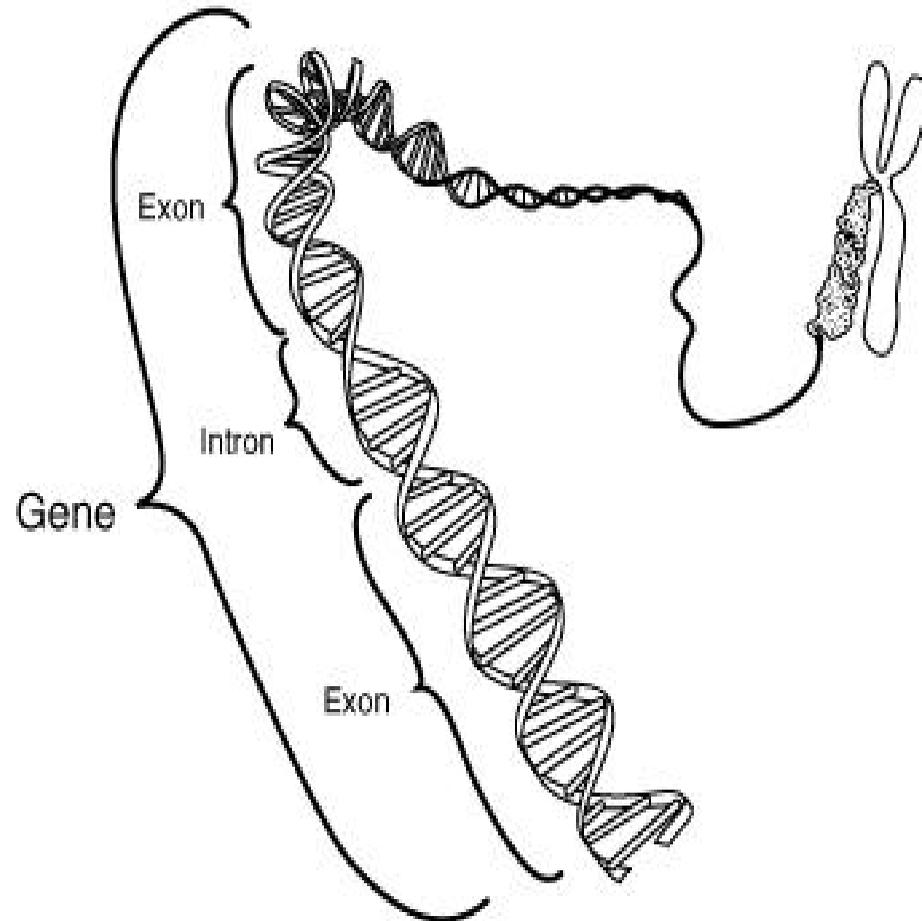


Ribosomal RNA

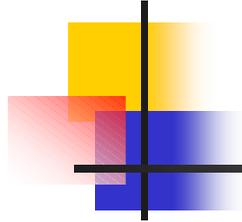




Gene



- A portion of the genome that encodes a specific product, such as a protein or enzyme or other macromolecule
- Example: the gene *stx1* encodes a shiga toxin from *E. coli* O157:H7

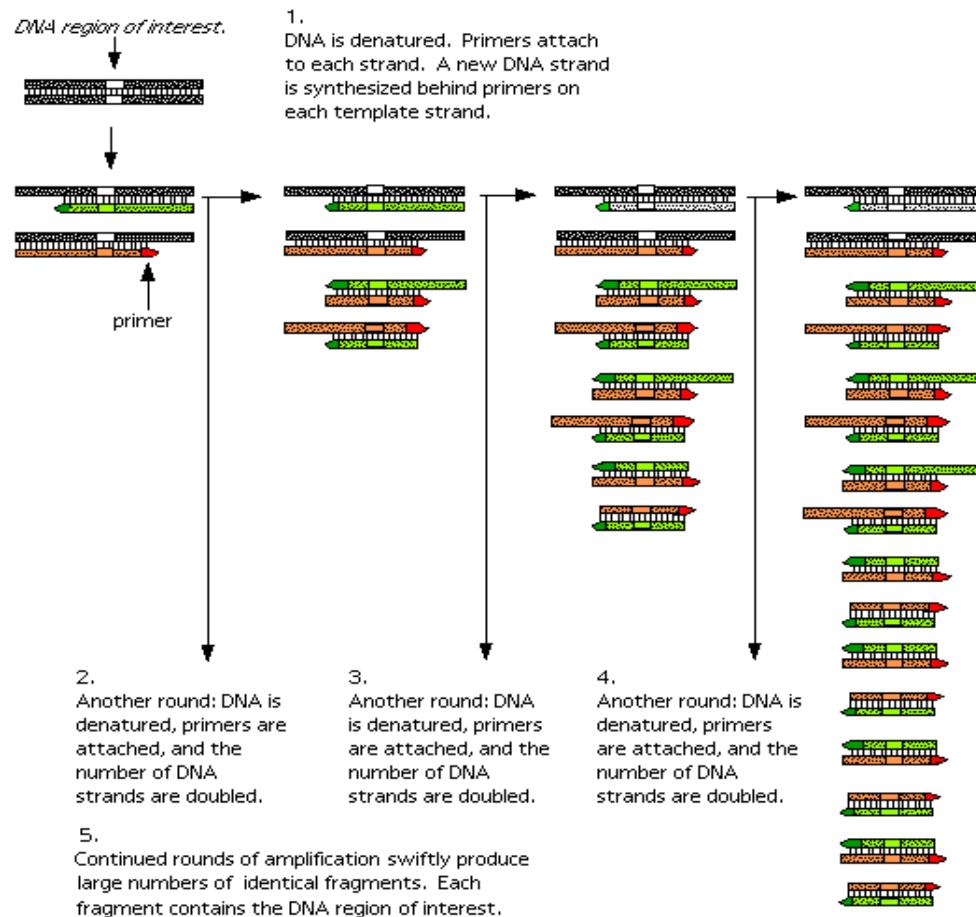


Primers

- 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

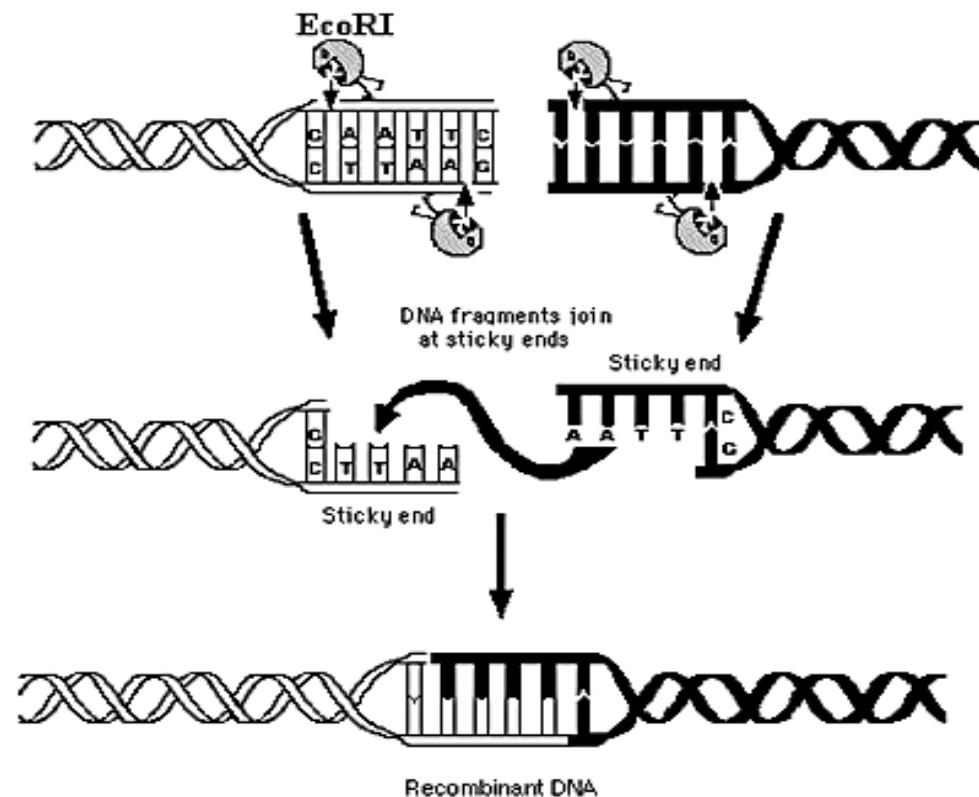
PCR

POLYMERASE CHAIN REACTION



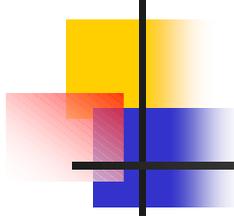
- Polymerase Chain Reaction
- Method developed by K. Mullis as a way to copy DNA *in vitro*
- One copy of a gene can be amplified 10^6 times so that it becomes "visible" by gel electrophoresis or by fluorescent assays

Restriction Enzyme



Restriction Enzyme Action of EcoRI

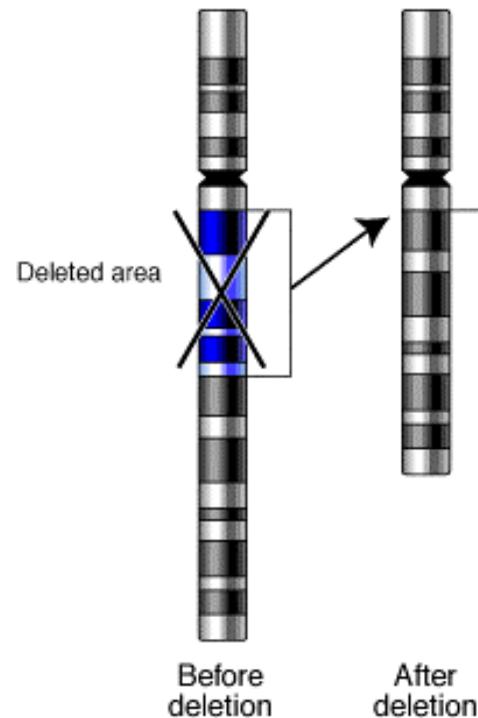
- Enzymes produced naturally by many strains of bacteria as a defense mechanism
- Enzymes cut DNA at specific combinations of A, G, C and T.
- Named systematically: EcoR1 is the first enzyme isolated from *Escherichia coli*



Fingerprinting

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

Mutation

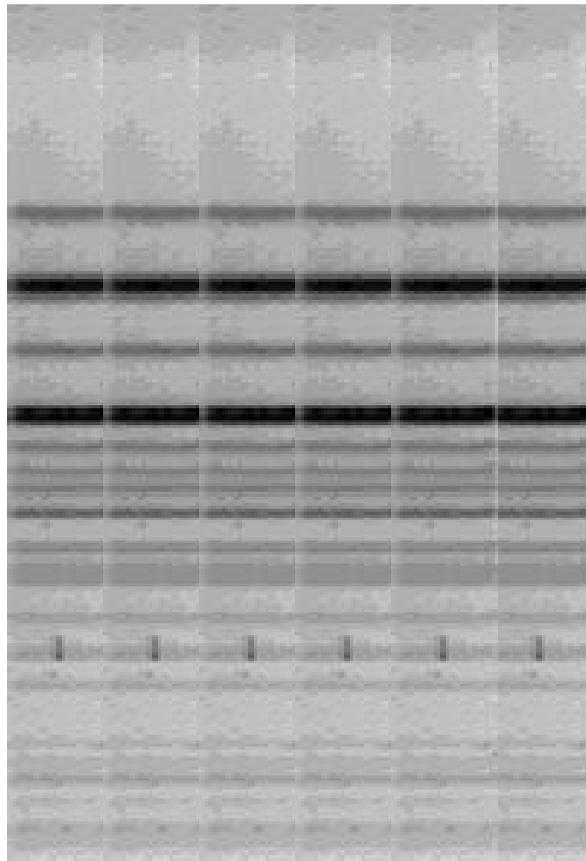


- A change in the nucleic acid sequence that may or may not be readily observed

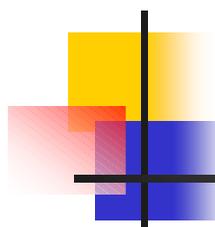
(lethal \longleftrightarrow neutral)

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

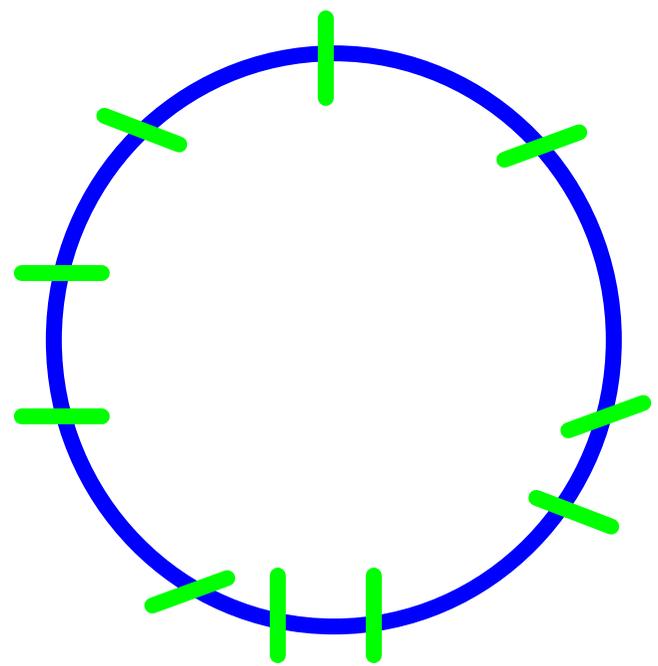
Pulsed-Field Gel Electrophoresis



- 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

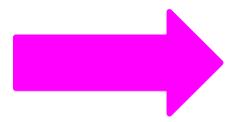


PFGE

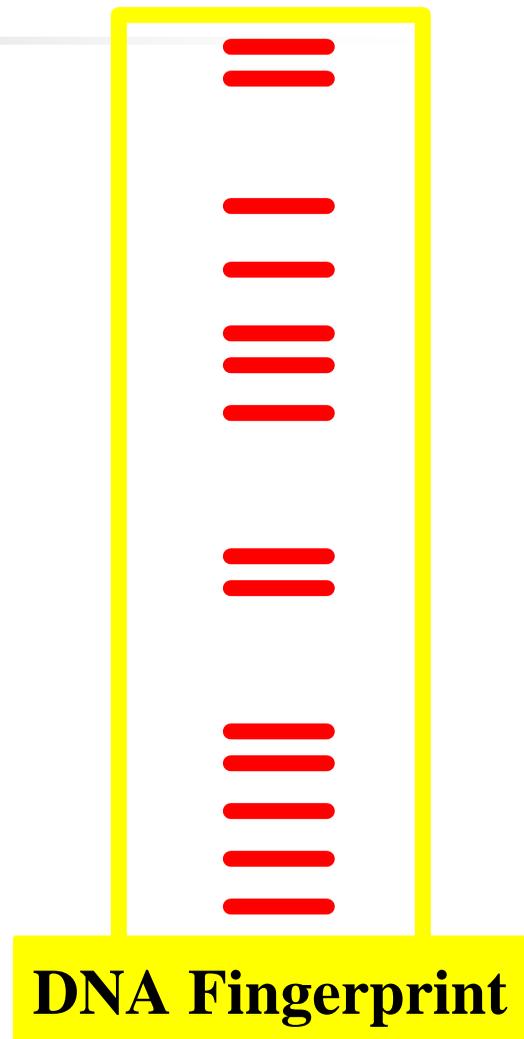


Bacterial Chromosome
With Rare Restriction Sites

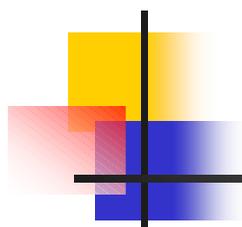
Enzyme Digest



Electrophoresis



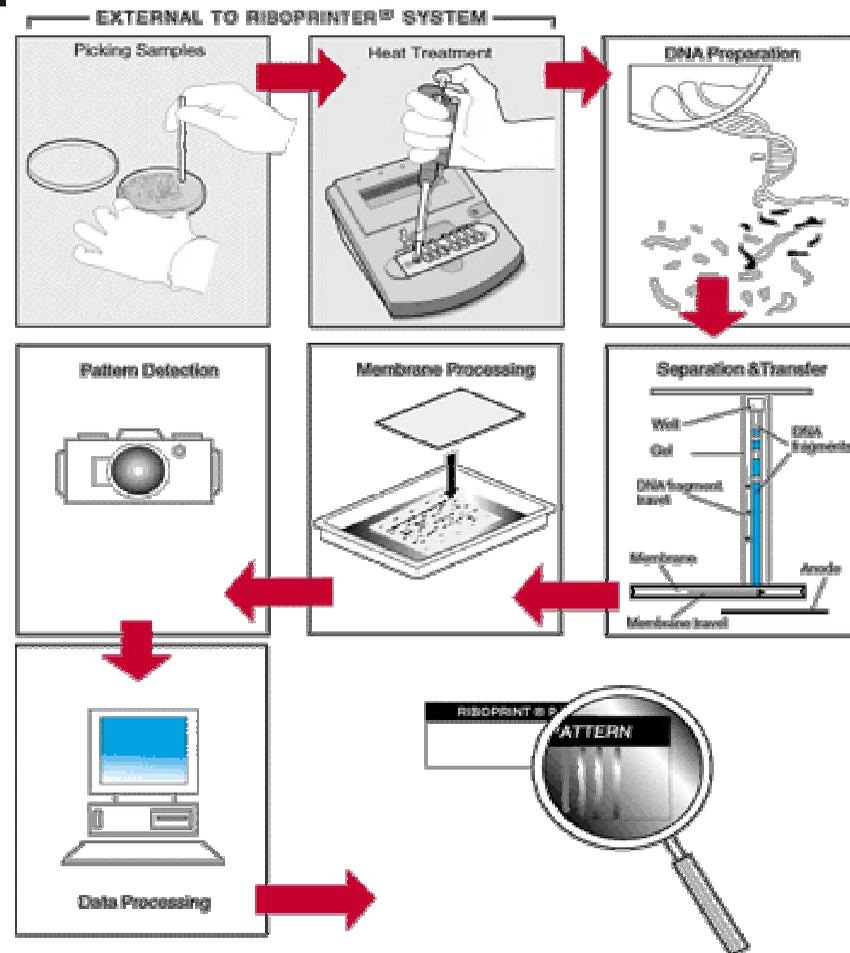
DNA Fingerprint



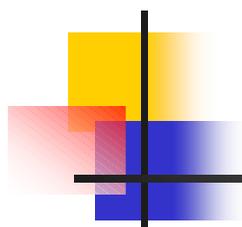
Restriction Fragment Length Polymorphism

- 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

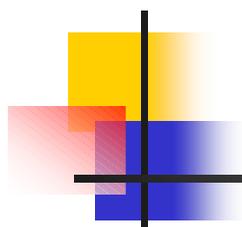


- Automated system available from Dupont-Qualicon (www.qualicon.com)
- Variation of RFLP, using primers to rRNA gene targets
- Chemiluminescent detection of banding patterns due to probe binding sites



Randomly Amplified Polymorphic DNA

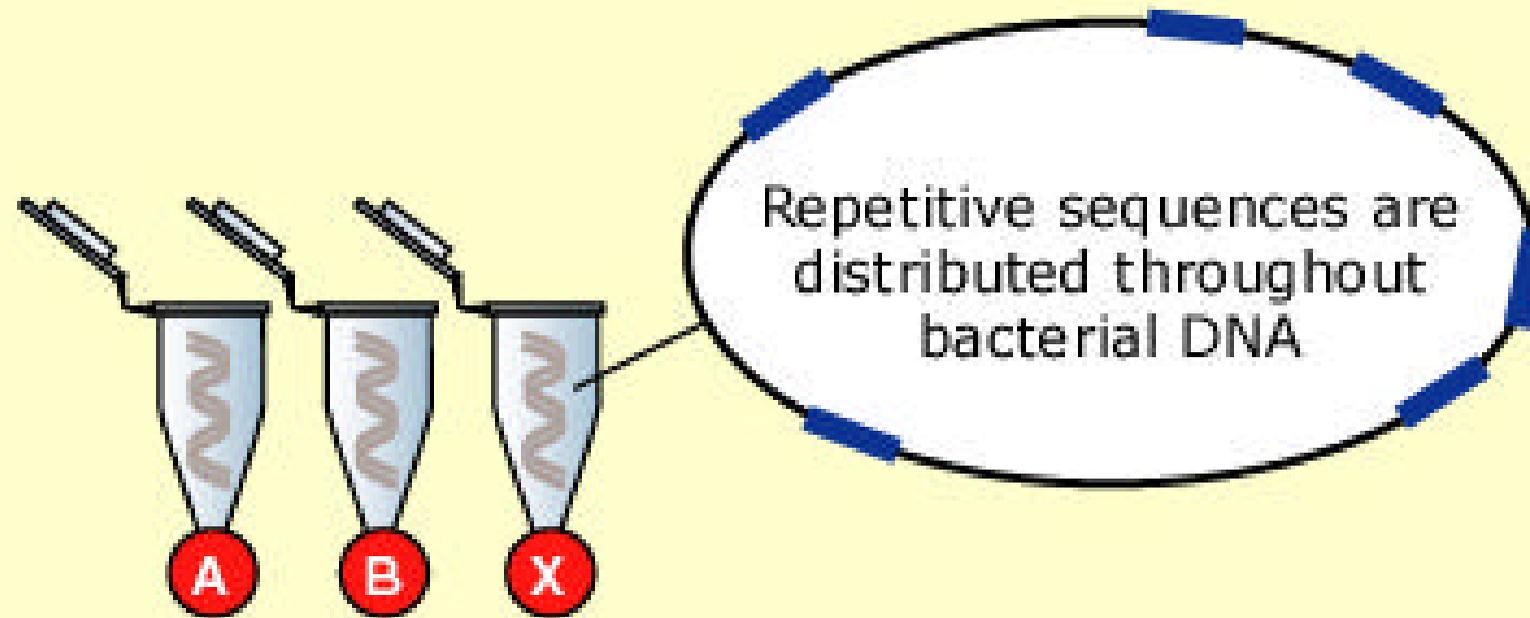
- RAPD uses short random sequence primers, 9-10 bases long
- Amplifies chromosomal DNA at low annealing temperatures
- Number and location of random primer sites vary for different strains



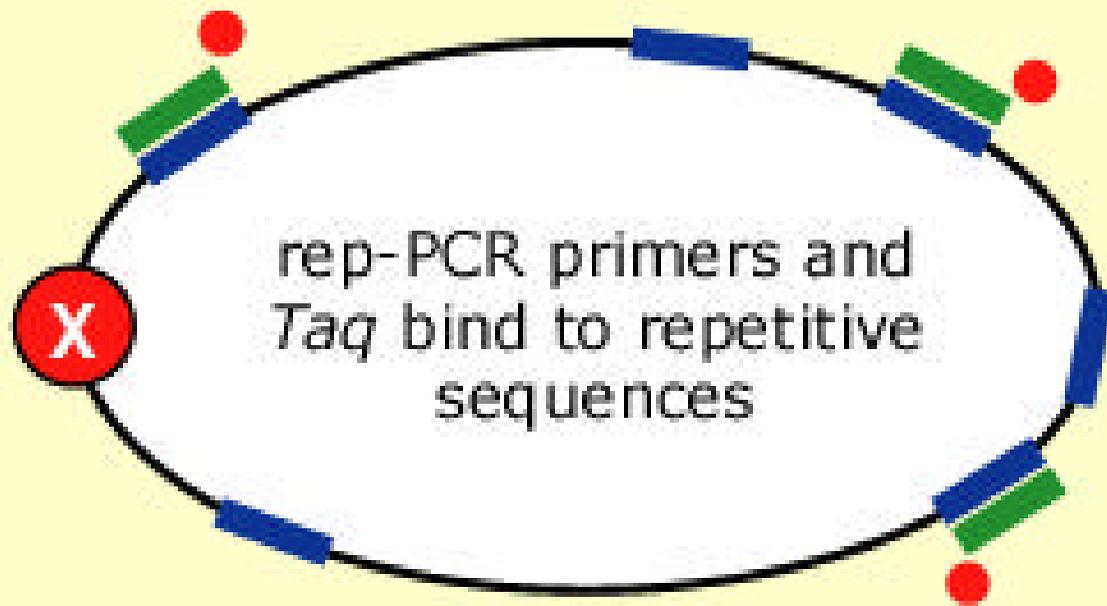
Repetitive Extragenic Palindromic Element PCR

- 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



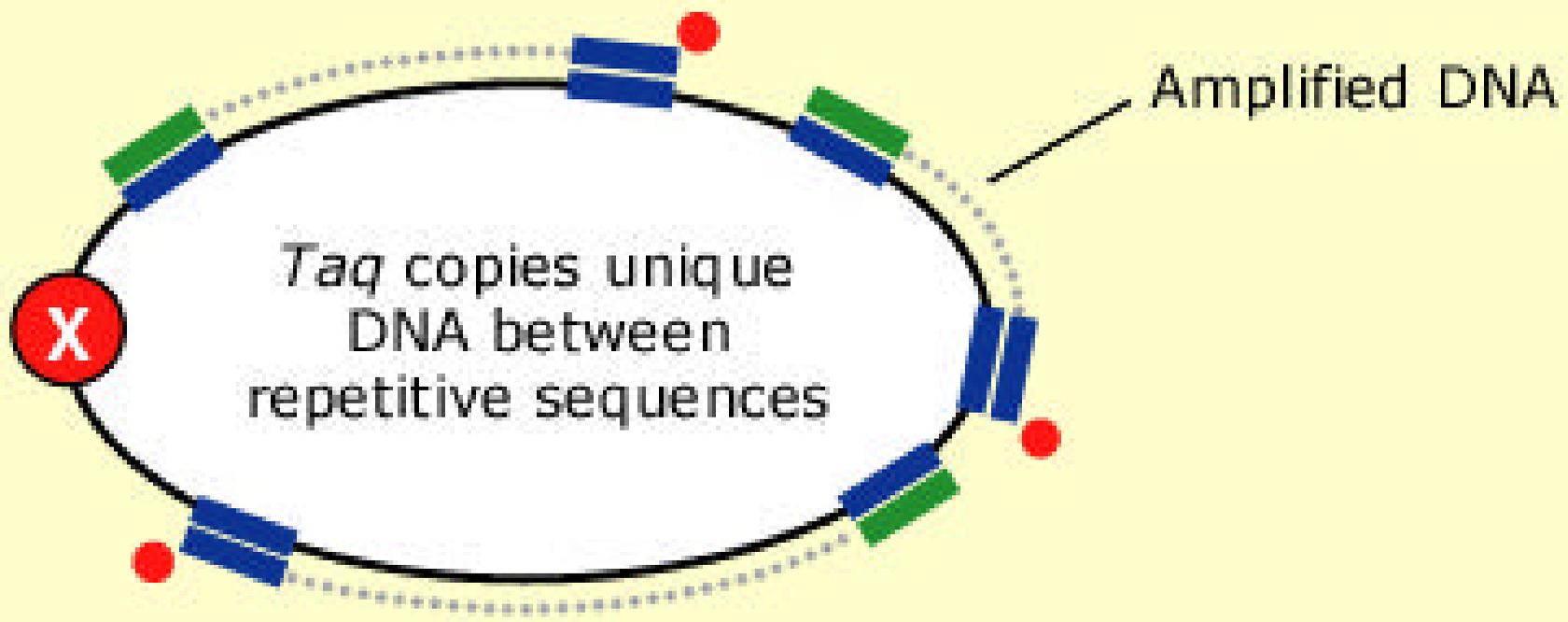
Rep-PCR



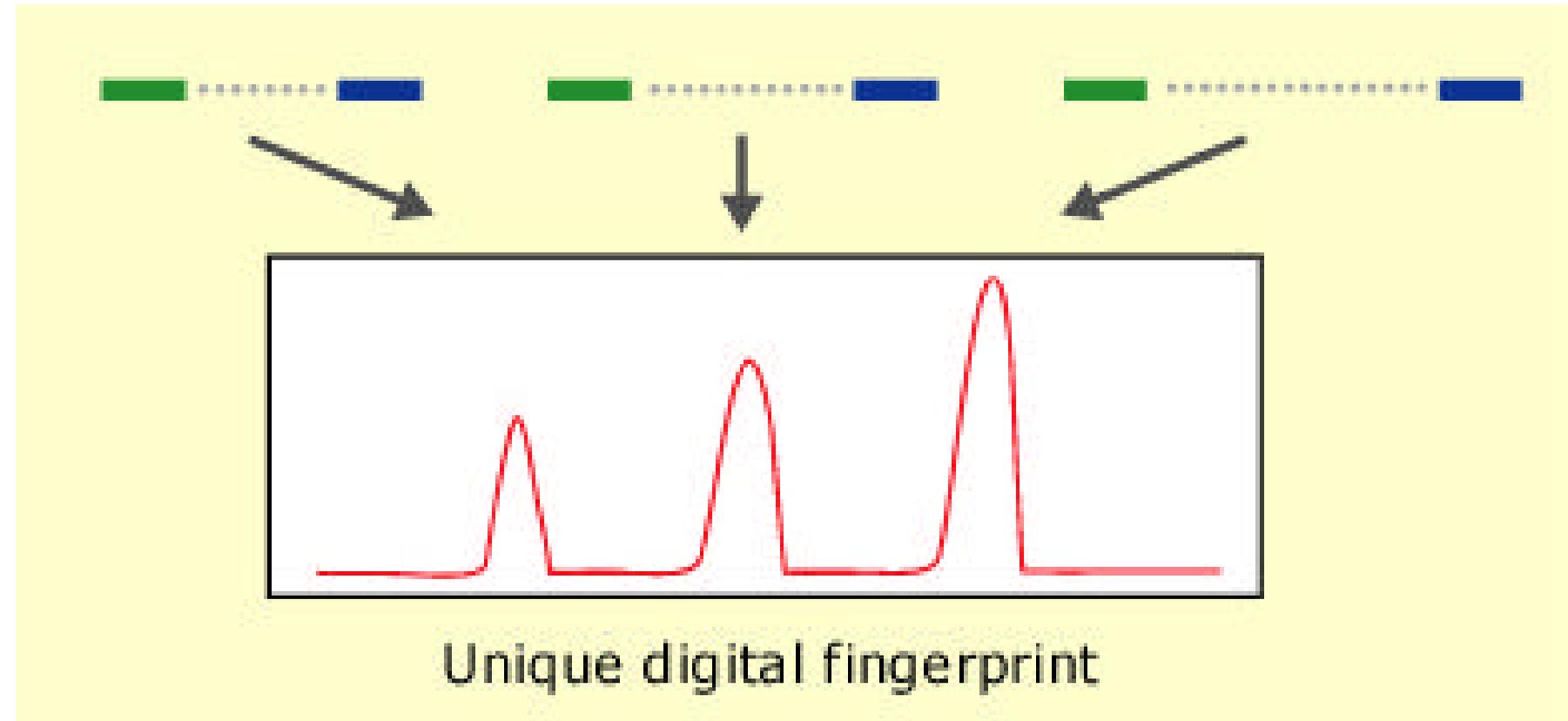
● *Taq* DNA polymerase

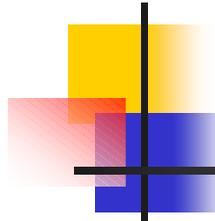
— rep-PCR primer

Rep-PCR

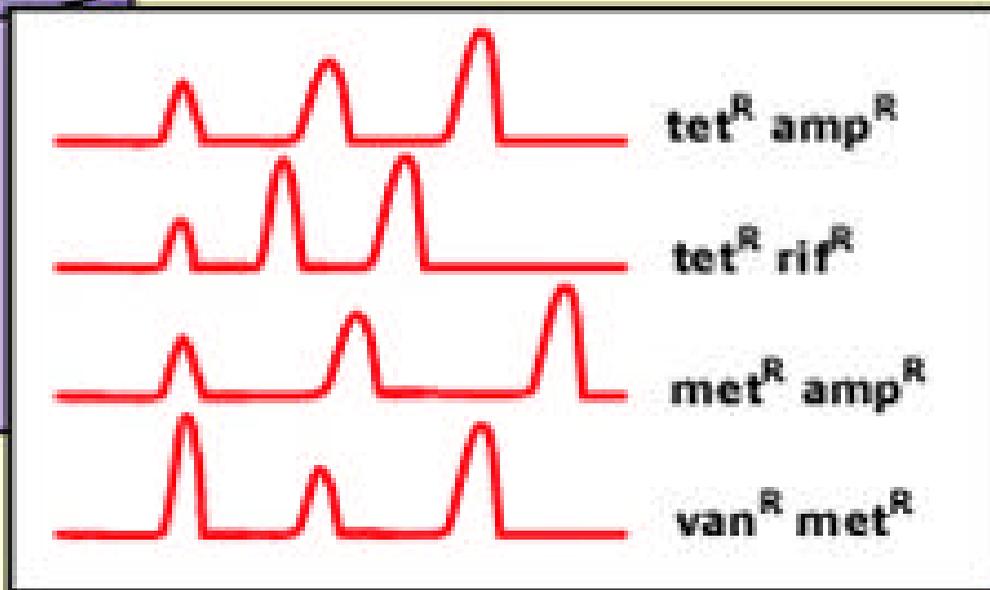
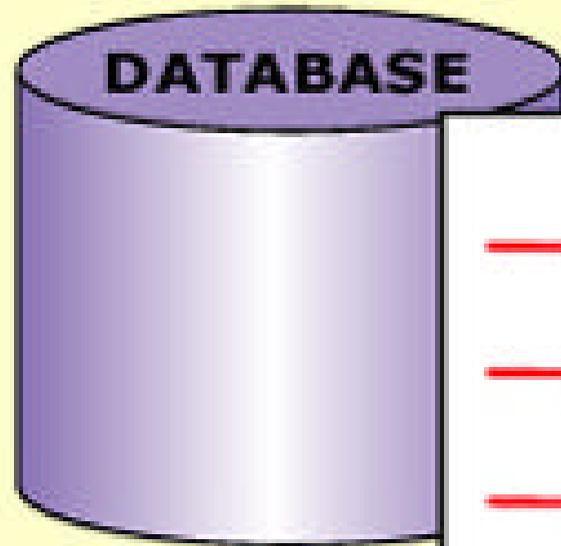


Rep-PCR

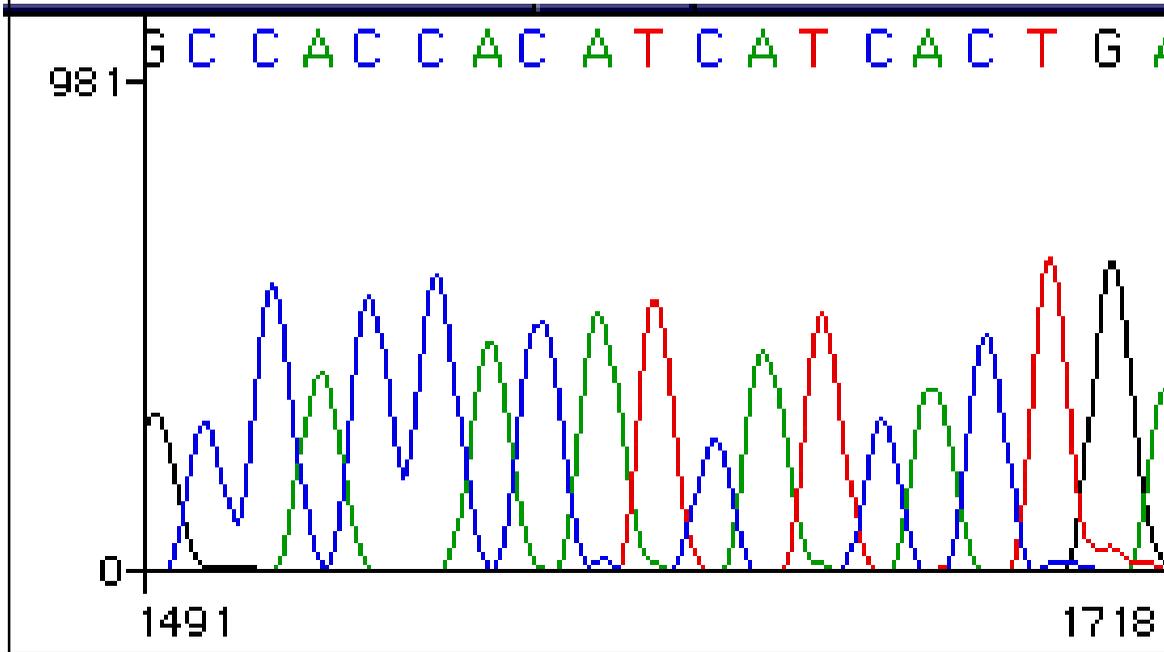




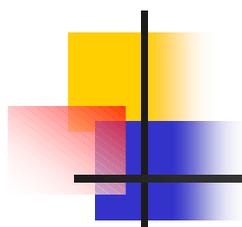
Rep-PCR



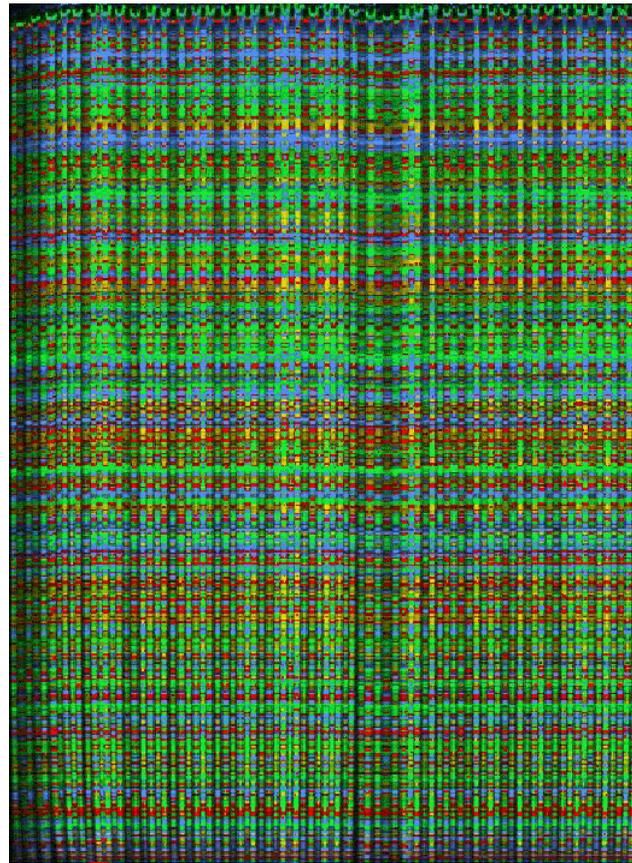
Next Generation Subtyping Methods



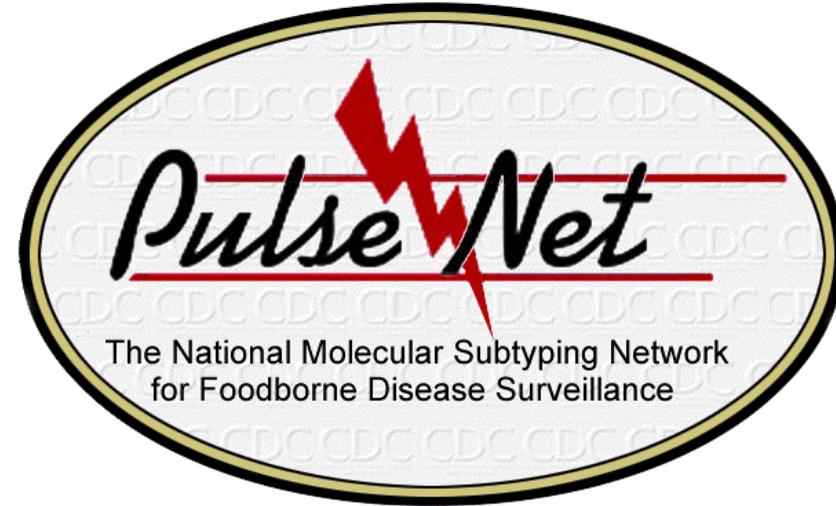
- DNA Sequencing
- MLST
- VNTR
- MLVA
- PATS
- Microarray



Multilocus Sequence Typing



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



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



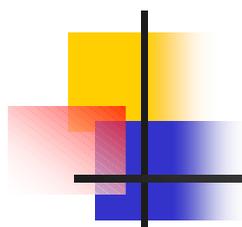
PulseNet



- 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



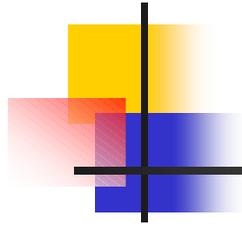
- Goals of national surveillance system are to identify clusters of foodborne disease and to assist in epidemiologic investigations
- Ideally this will limit foodborne outbreaks by identifying sources of contamination and by raising awareness of food safety issues



Escherichia coli O157:H7



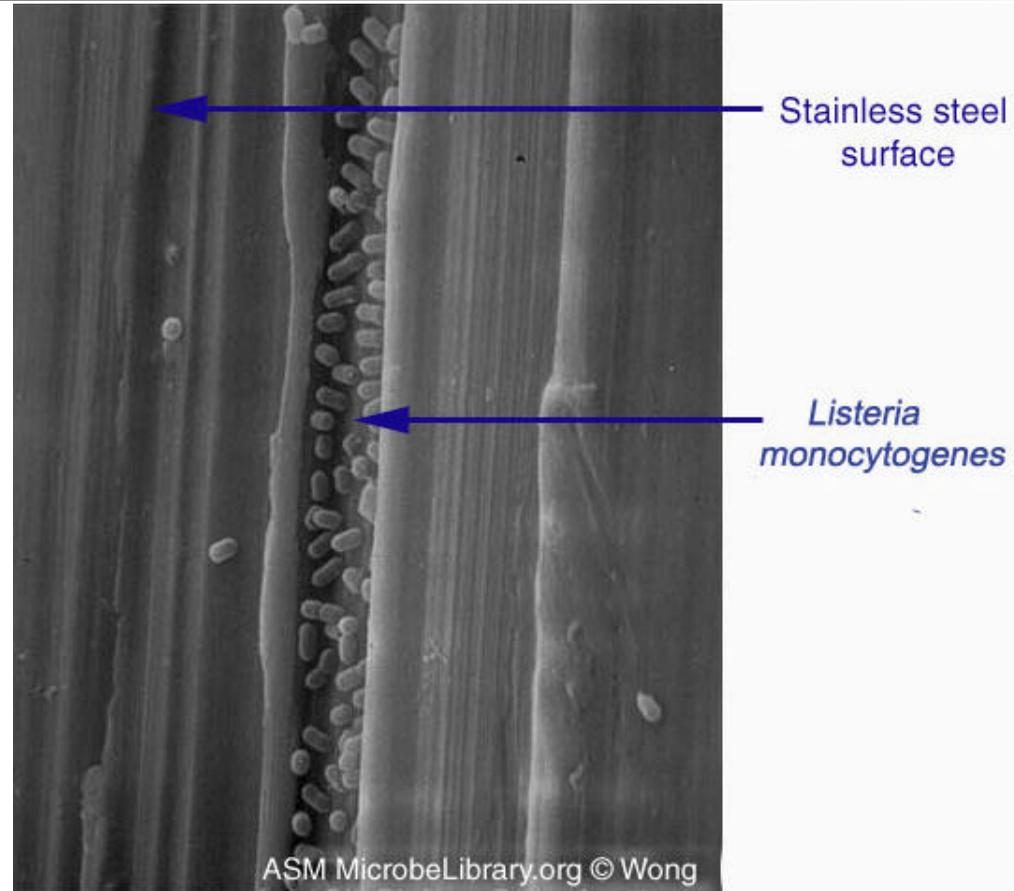
SEM of *Escherichia coli* ©Scott Kachlany, author. Licensed for Use, ASM MicrobeLibrary



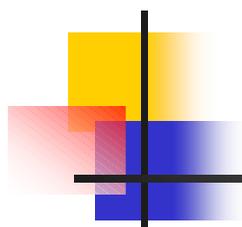
E. coli O157:H7

- 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

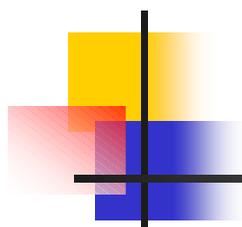


SEM of *Listeria monocytogenes* ©Amy C. Lee Wong, author. Licensed for Use, ASM MicrobeLibrary



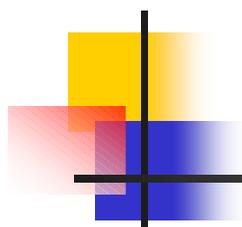
Listeria monocytogenes

- Gram positive rod, intracellular pathogen
- Causes sepsis or meningitis in immunocompromised 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



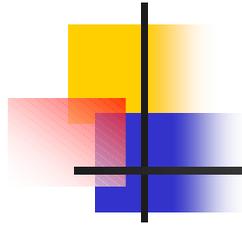
Salmonella enterica

- 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



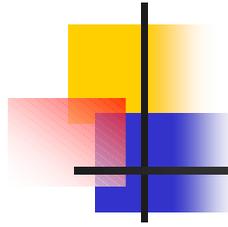
Campylobacter jejuni

- 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

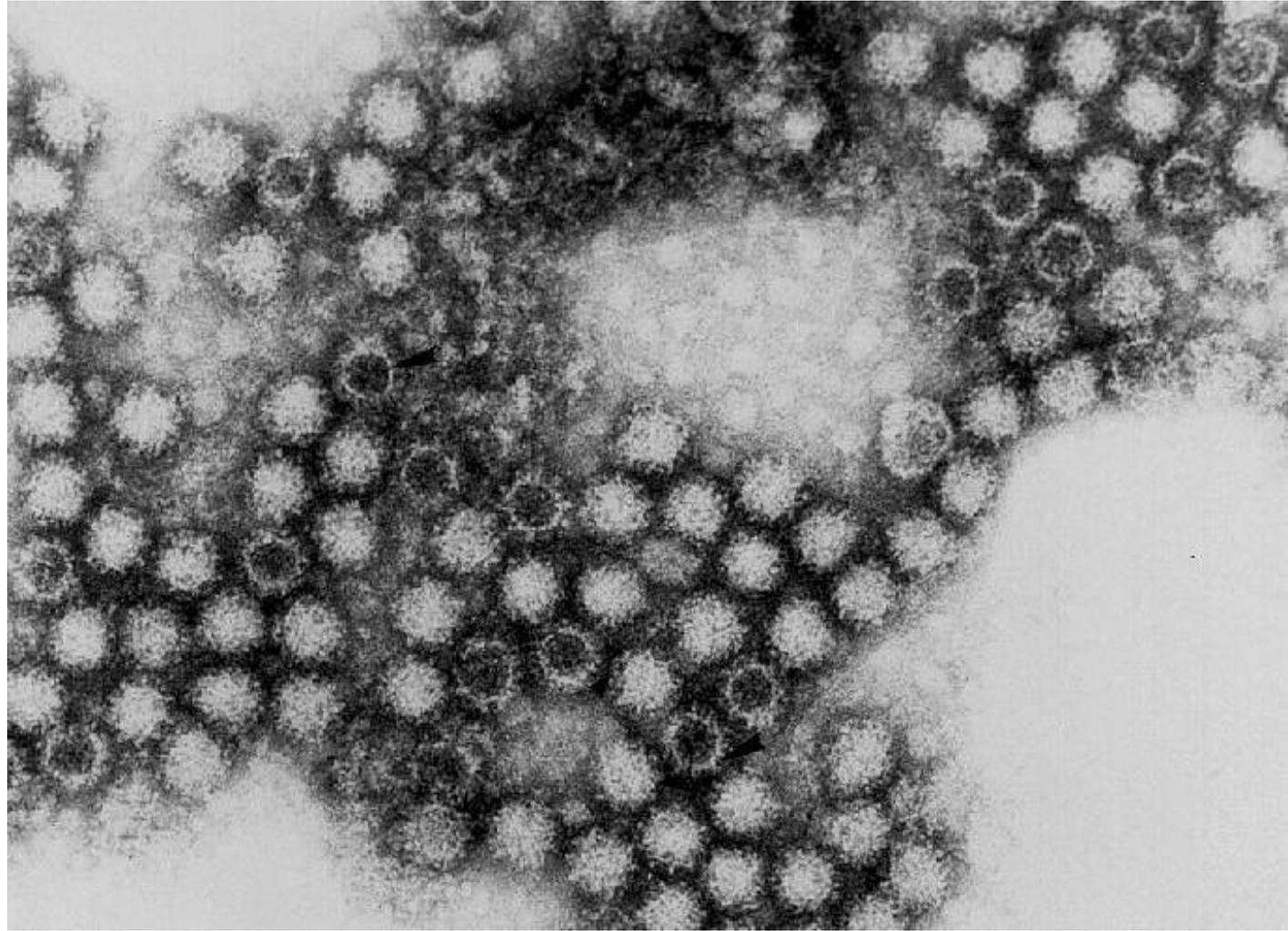


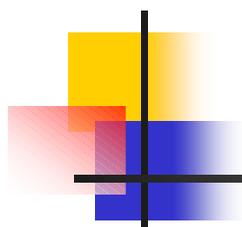
Shigella sonnei

- 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



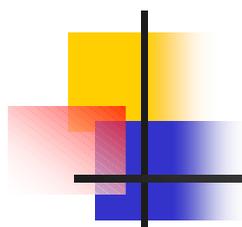
Calicivirus





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

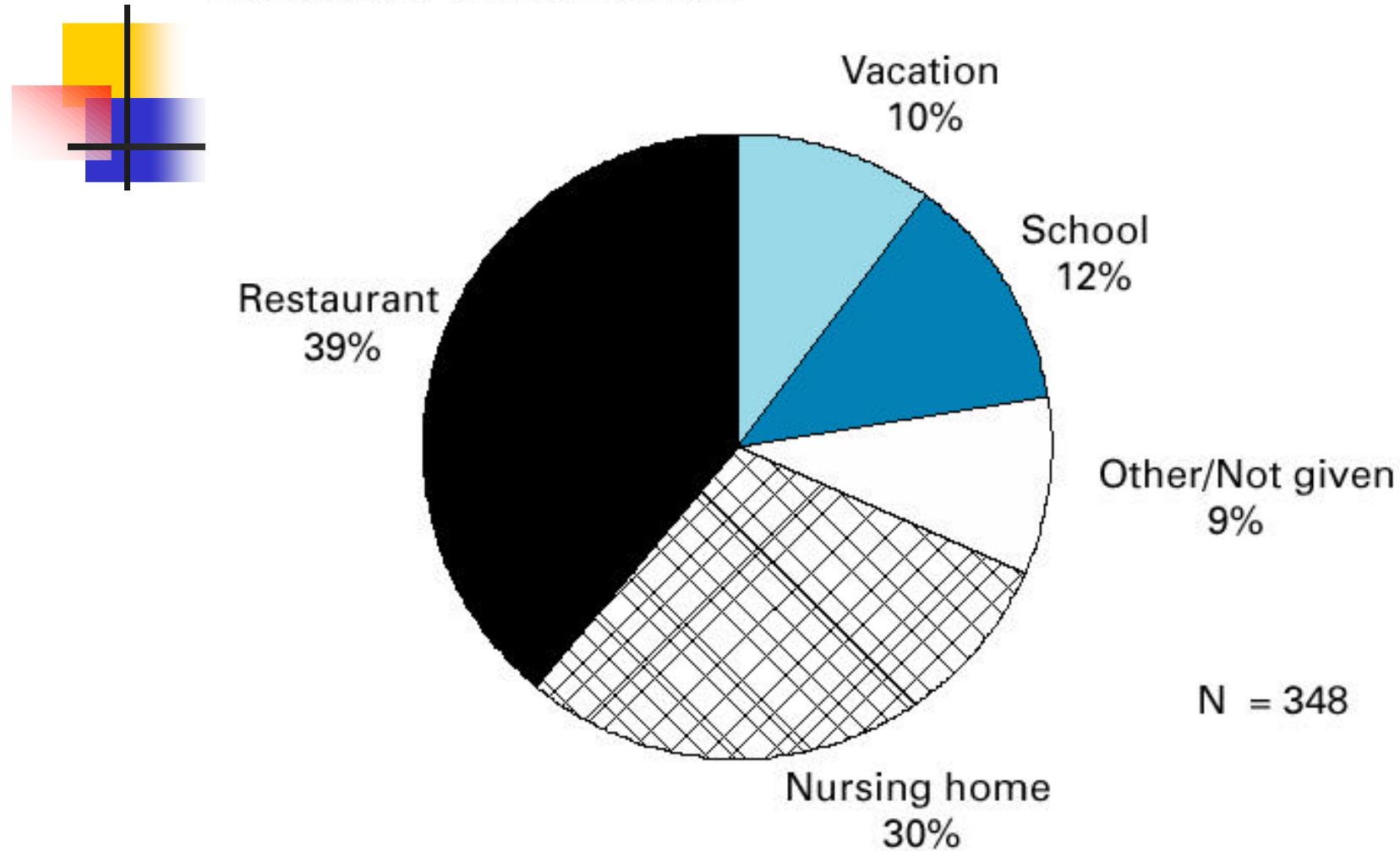


MMWR 50(RR09): 1-18, 2001

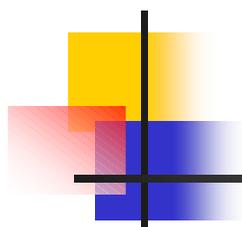
TABLE 1. Characteristics of "Norwalk-like viruses" that facilitate their spread during epidemics

Characteristic	Observation	Consequences
Low infectious dose	$<10^2$ viral particles	Permits droplet or person-to-person spread, secondary spread, or spread by foodhandlers
Prolonged asymptomatic shedding	≤ 2 weeks	Increased risk for secondary spread or problems with control regarding foodhandlers
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 composite 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*

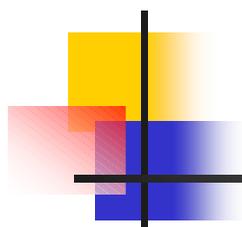


***Source:** Fankhauser RL, Noel JS, Monroe SS, Ando T, Glass RI. Molecular epidemiology of "Norwalk-like viruses" in outbreaks of gastroenteritis in the United States. *J Infect Dis* 1998;178:1571–8; and CDC, unpublished data, 1997–2000.



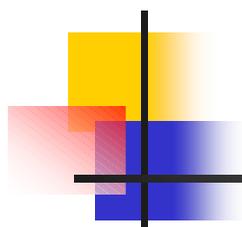
E. coli O157:H7 outbreak, Robeson County, NC 2001

- 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

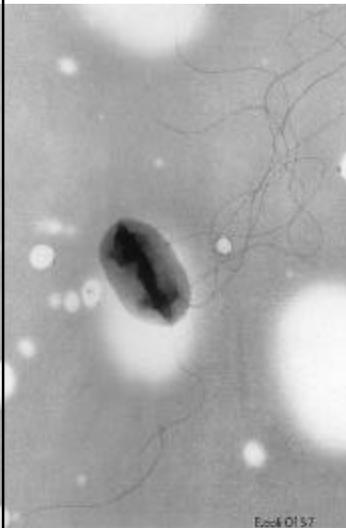


E. coli O157:H7 outbreak

- 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

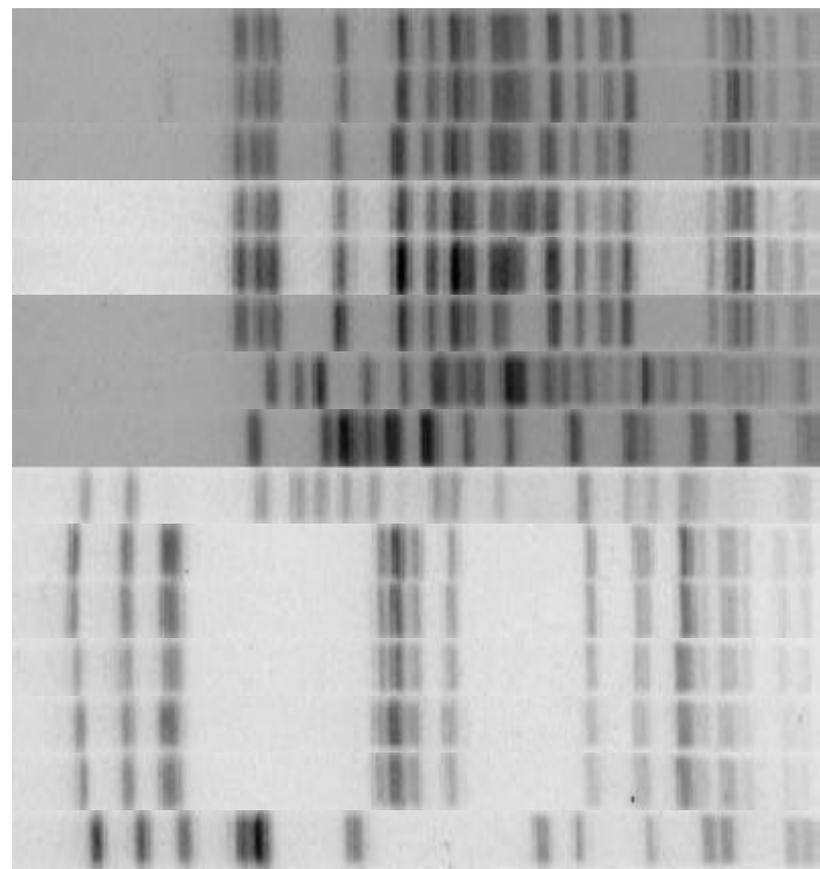
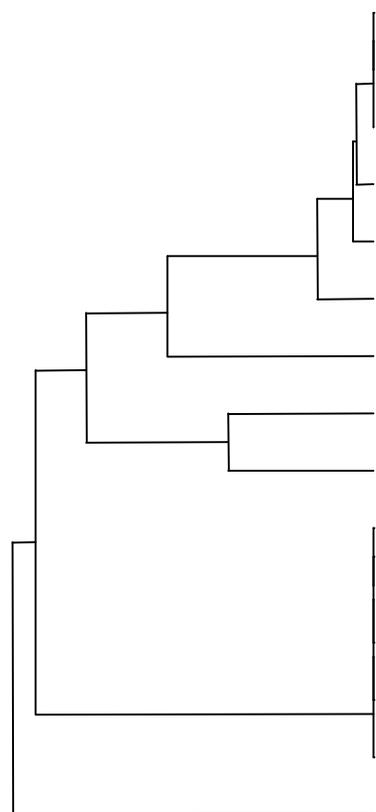
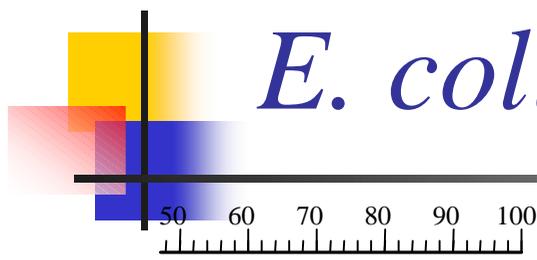


E. coli O157:H7 outbreak



- 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

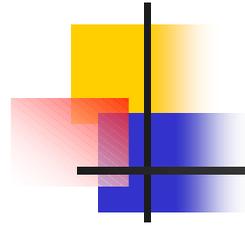
E. coli O157:H7 PFGE Patterns



A
A
A
A1
A2
A3
B
C

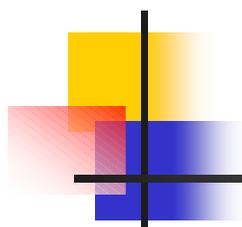
XbaI patterns

AvrII patterns



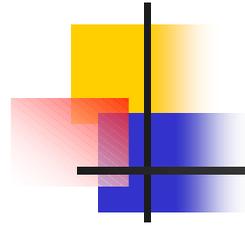
Recommendations

- 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



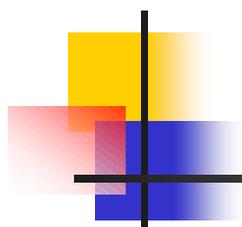
L. monocytogenes outbreak, Forsyth County, NC 2000

- 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

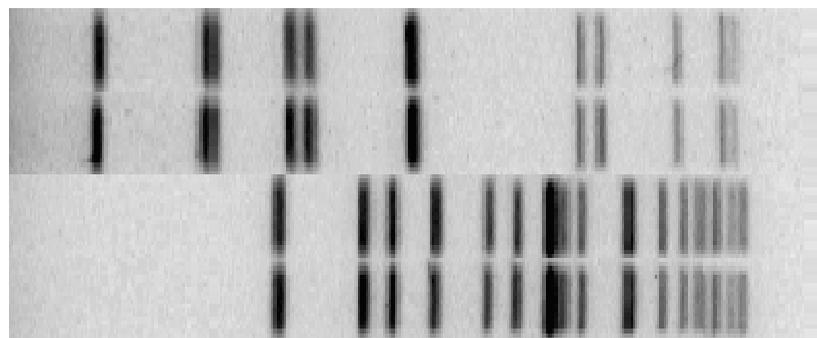
- Investigation of these cases revealed that Mexican-style soft cheese was made in private homes from raw milk
- Home-made cheese sold door-to-door or in parking lots
- Found unlabeled home-made cheese in local Latino grocery stores



L. monocytogenes PFGE Patterns



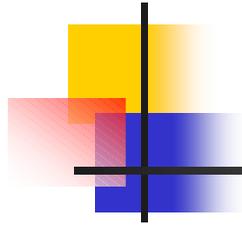
Serotype 4b



Ascl digest

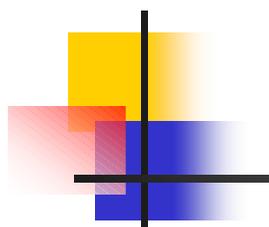
Apal digest

Ribotyping patterns were indistinguishable: DUP1042

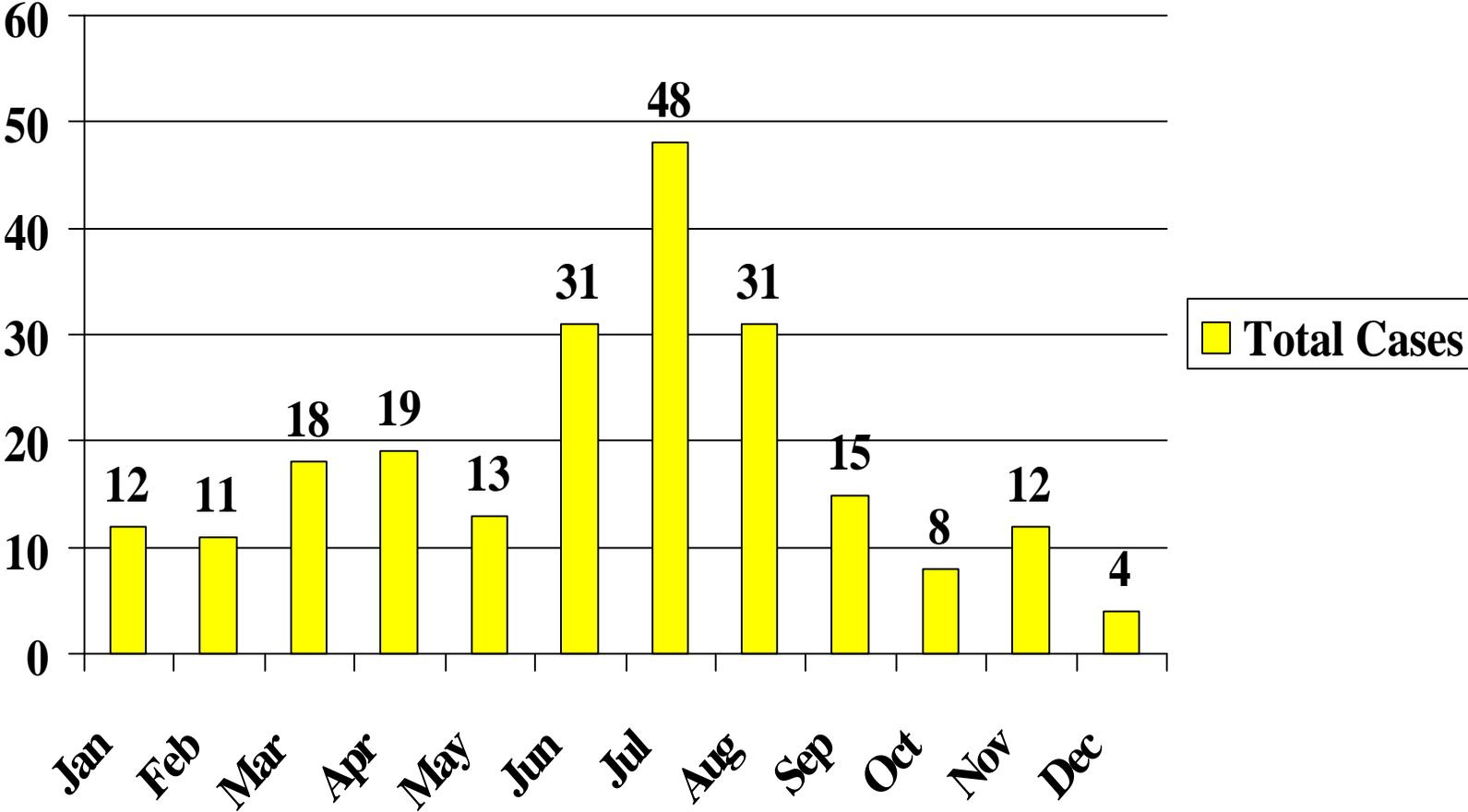


Recommendations

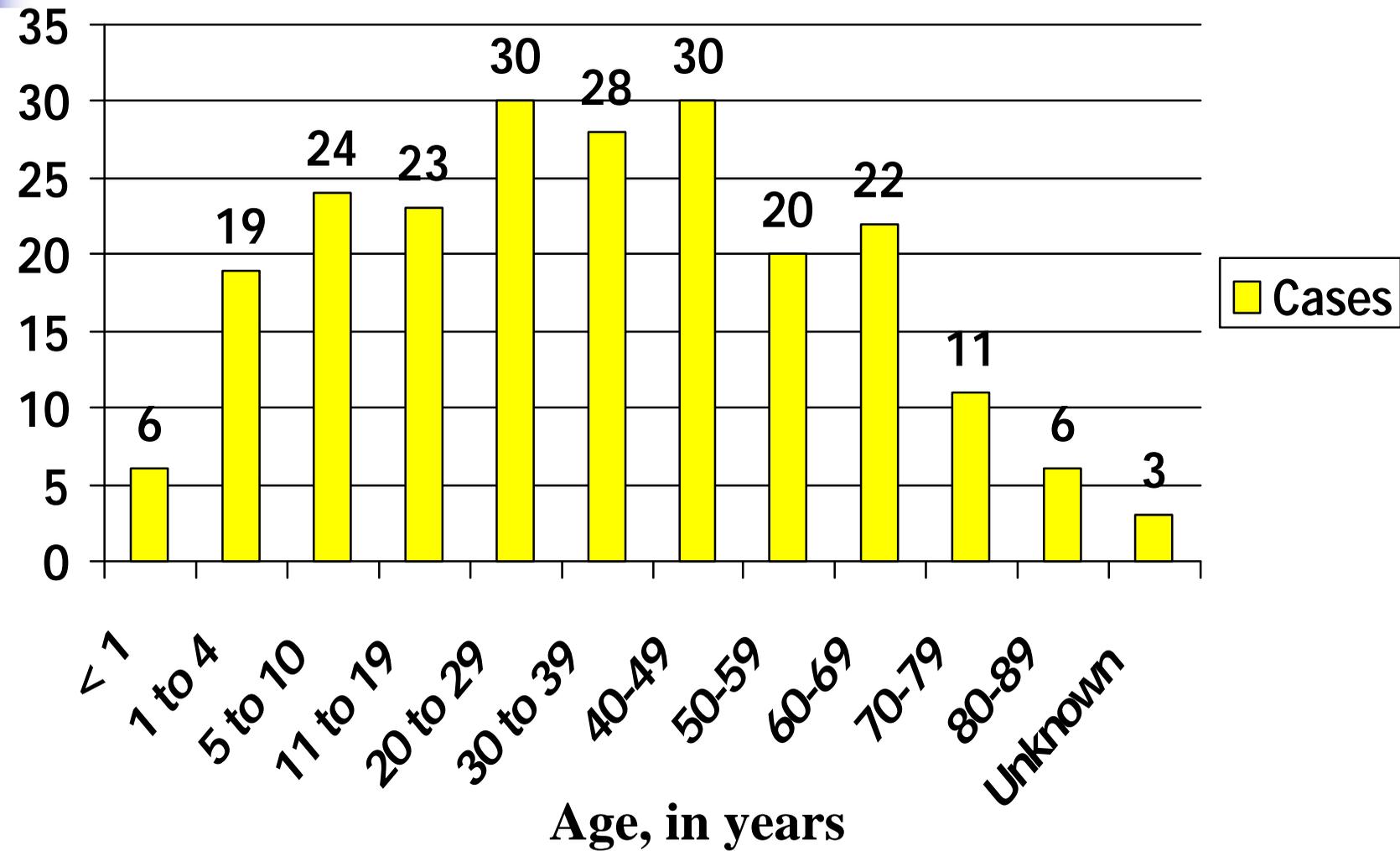
- Limit sale of raw milk products to regulated processors
- Educate hispanic communities about the dangers of eating unpasteurized cheeses while pregnant
- Make listeriosis a reportable illness in North Carolina
- *MMWR* 50(26): 560-562, July 6, 2001



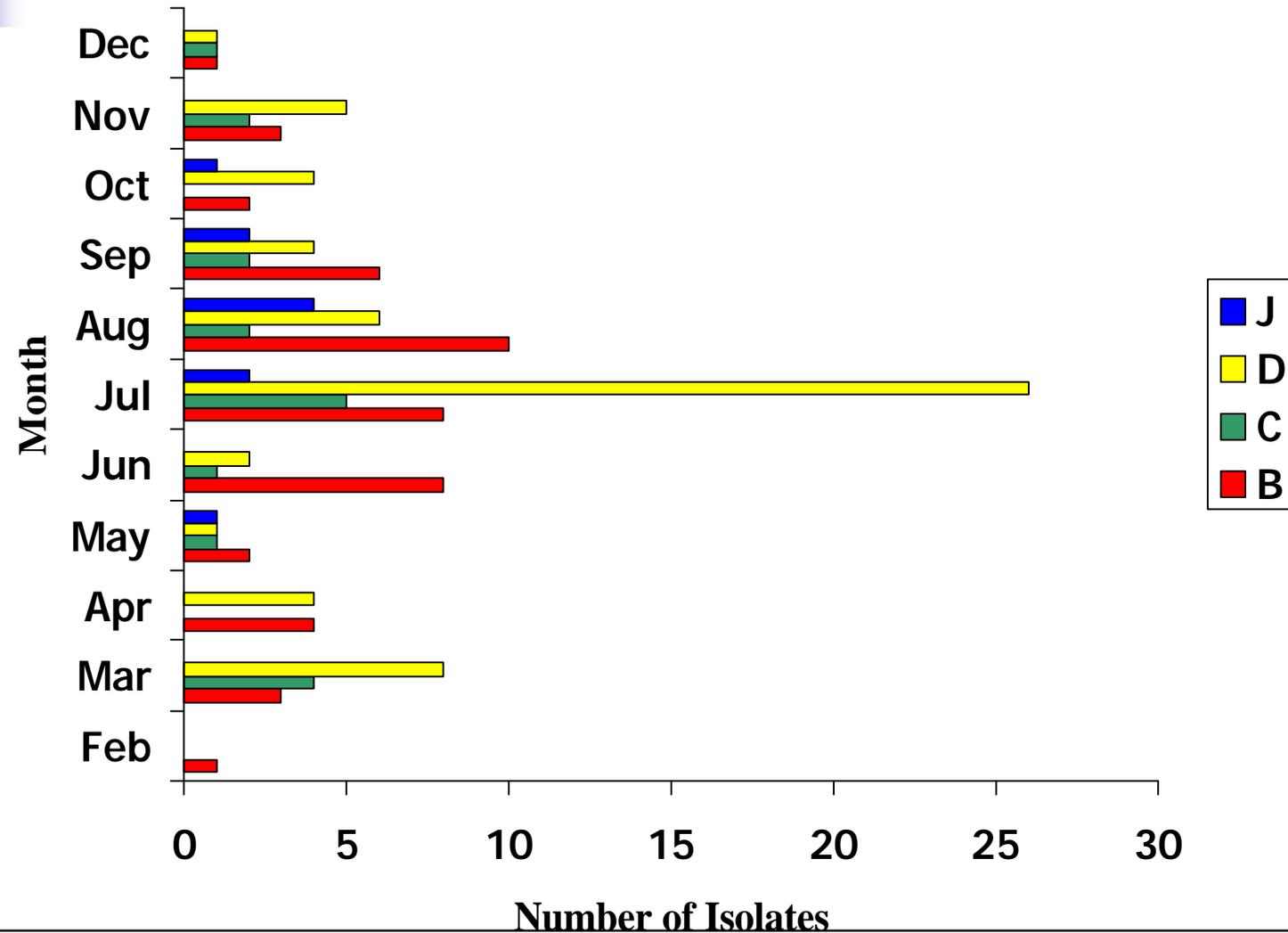
North Carolina *Salmonella* Enteritidis Case Numbers by Month, 2001



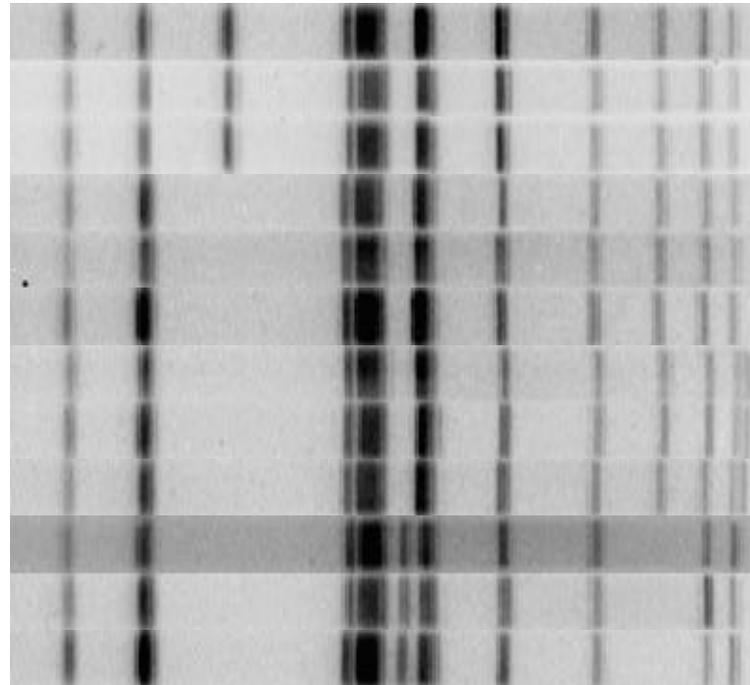
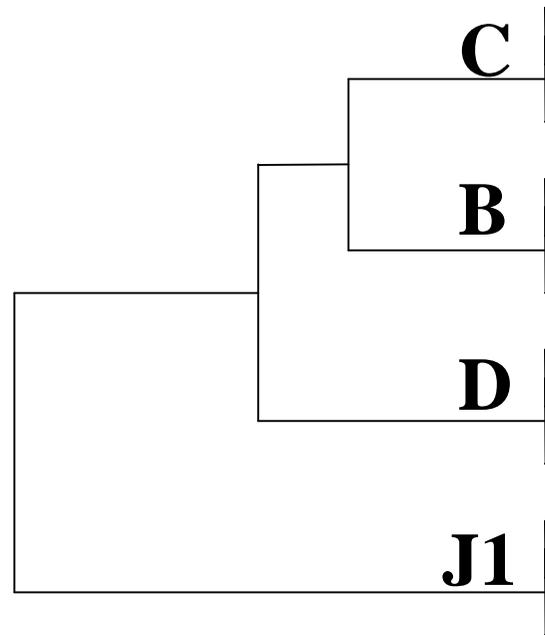
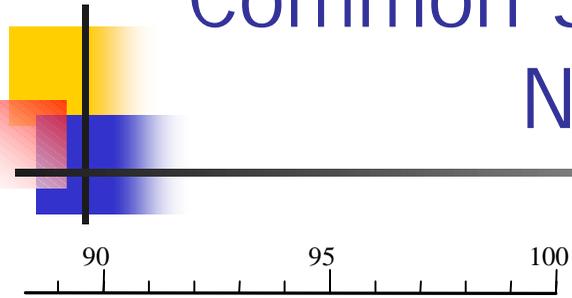
Age Distribution of *S. Enteritidis* cases in North Carolina, 2001

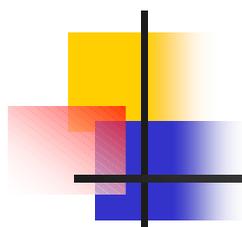


Number of Isolates with Common *S. Enteritidis* PFGE Patterns, 2001



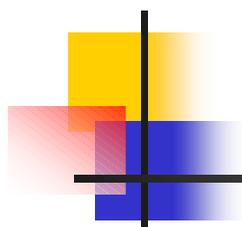
Common *S. Enteritidis* PFGE Patterns, North Carolina 2001





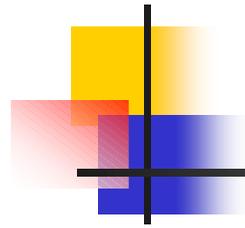
Conclusions

- 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*



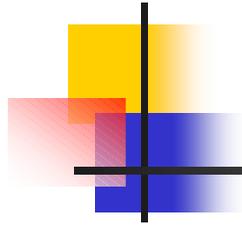
Norwalk-like virus outbreak, Texas 1998

- 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?

