

ABSTRACT

Title of Dissertation: DETECTION AND CHARACTERIZATION
 OF *ESCHERICHIA COLI* O157:H7 AND
 SALMONELLA IN FOOD

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Escherichia coli O157:H7 and *Salmonella* are among the most important foodborne pathogens that cause millions cases of infections and hundreds deaths each year in the United States. Beef and poultry products are frequently recognized transmission media for these two organisms. Rapid detection and isolation methods applied to beef or chicken products are expected for these two bacteria. A rapid sample preparation method for *E. coli* O157:H7 detection by PCR method in ground beef samples was developed by combining different techniques, including filtration, centrifugation, enzyme digestion, and DNA extraction. The detection limit of this method was 10^3 cells/g without enrichment, and 10^0 cells/g can be detected after 6 h en-richment. For *Salmonella*, a poultry specific isolation method was modified from

the USDA/FSIS manual by considering the specific characteristics of poultry products. Higher than 95% of the suspect colonies isolated by using the modified method were confirmed as *Salmonella* by PCR/API 20 E tests. This method was applied on retail organic and conventional chicken samples for *Salmonella* isolation. All *Salmonella* isolates were further characterized by serotyping, PFGE and antibiotics susceptibility tests. The results indicated that organic and conventional chicken samples were frequently contaminated with *Salmonella*, and that *Salmonella* from organic chicken were more susceptible to antimicrobials commonly used in human and veterinary medicine.

High acid resistance capability is another unique characteristic of *E. coli* O157:H7 which is correlated with low infection dose of this pathogen. Survival mechanism of *E. coli* O157:H7 cells in gastric juice or acidified LB (pH 2.5) was studied, it was found that the limited availability of glutamate and/or arginine creates an illusion of cell-density-dependent acid sensitive phenotype of *E. coli* O157:H7 during acid-challenge.

DETECTION AND CHARACTERIZATION OF
ESCHERICHIA COLI O157:H7 AND *SALMONELLA* IN FOOD

by

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LIST OF ABBREVIATIONS

ACSSuT	Ampicillin-chloramphenicol-streptomycin-sulfonamides-tetracycline
<i>AdiA</i>	Arginine decarboxylase system
AHI	Animal Health Institute
AHLs	<i>N</i> -acyl-L-homoserine lactones
AMI	Amikacin
AMP	Ampicillin
API 20 E	identification system for <i>Enterobacteriaceae</i>
AUG	Amoxicillin-clavulanic acid
AXO	Ceftriaxone
BAM	The FDA Bacteriological Analytical Manual
BGS	Brilliant green sulfa
BPW	Buffered peptone water
BS	Bismuth sulfite
CEP	Cephalothin
CFU	Colony forming unit
CHL	Chloramphenicol
CIP	Ciprofloxacin
COT	Trimethoprim/Sulfamethoxazole
DMLIA	Double modified lysine iron
EHEC	Enterohemorrhagic <i>E. coli</i>
ELISA	Enzyme-linked immunosorbent assay

FDA	Food and Drug Administration
FOX	Cefoxitin
FSIS	Food Safety Inspection Service
<i>gadABC</i>	Glutamate decarboxylase system
GEN	Gentamicin
HE	Hektoen enteric
KAN	Kanamycin
LB	Luria-Bertani
MIC	Minimal inhibitory concentration
NA	Nalidixic acid
NARMS	National Antimicrobial Resistance Monitoring Systems
NCCLS	National Committee Clinical Laboratory Standards
O.D. ₆₀₀	Optical density at 600 nm
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
<i>RpoS</i>	Alternative sigma-transcription factor
RV broth	Rappaport-Vassiliadis broth
SMX	Sulfamethoxazole
STEC	Shiga toxin-producing <i>E. coli</i>
STR	Streptomycin
Stx1	Shiga-like toxin one
Stx2	Shiga-like toxin two
TET	Tetracycline

TIO	Ceftiofur
TSI	Triple sugar iron
TT	Tetrathionate
USDA	United States Department of Agriculture
XLD	Xylose lysine desoxycholate
XLT4	Xylose lysine tergitol 4

Chapter 1 Introduction

There have been over 200 microbial pathogens identified to be associated with foodborne illnesses, including bacteria, viruses, and parasites (56). Each year, these pathogens cause an estimate of 76 million cases of foodborne illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States. The top three foodborne pathogens are Norwalk-like virus, *Campylobacter* and nontyphoidal *Salmonella*, which account for 90% of cases of foodborne illnesses in the United States (Table 1.1). Food-related deaths are caused mostly by six pathogens: *Salmonella* (31%), *Listeria* (28%), *Toxoplasma* (21%), Norwalk-like viruses (7%), *Campylobacter* (5%), and *Escherichia coli* O157:H7 (3%) (39). The epidemiology of foodborne diseases has changed in the last three decades partly because newly recognized pathogens emerge and well-recognized pathogens increase or become associated with food or new food vehicles. Among the 10 most common foodborne pathogens, four were recognized only in the last 30 years which account for 81% of foodborne illnesses of known causes (Table 1.1.). Several pathogens have been newly emerged to be important foodborne pathogens, including *E. coli* O157:H7 and other enterohemorrhagic *E. coli*, and multidrug resistant *Salmonella* such as *S. Typhimurium* DT 104 and *S. Newport*. This chapter will focus on the major characteristics of these pathogens, their significance as foodborne pathogens, and methods for detecting such pathogens in food.

Table 1.1. Annually estimated food-borne illnesses, hospitalizations and deaths caused by major known food-borne pathogens, United States (39)

Disease or agent	Illnesses		Hospitalizations		Deaths	
	# of cases	% of total	# of cases	% of total	# of cases	% of total
		foodborne		foodborne		foodborne
<i>Campylobacter</i> spp.*	1,963,141	14.2	10539	17.3	99	5.5
<i>Salmonella</i> , nontyphoidal	1,341,873	9.7	15608	25.6	553	30.6
<i>Clostridium perfringens</i>	248,520	1.8	41	0.1	7	0.4
<i>Staphylococcus</i> food poisoning	185,060	1.3	1753	2.9	2	0.1
<i>Listeria monocytogenes</i> *	2,493	<0.1	2298	3.8	499	27.6
<i>E. coli</i> O157:H7*	62,458	0.5	1843	3.0	52	2.9
<i>Shigella</i> spp.	89,648	0.6	1246	2.0	14	0.8
Giardia lamblia	200,000	1.4	500	0.8	1	0.1
Norwalk-like viruses*	9,200,000	66.6	20000	32.9	124	6.9

*: Pathogens have emerged in the last 30 years.

***E. coli* O157:H7**

According to the modified Kauffman scheme, *E. coli* are serotyped on the basis of their O (somatic, polysaccharide chain), H (flagellar), and K (capsular) surface antigen profiles (18). Over 200 different O antigens and approximately 30 H antigens are recognized currently (30). A specific combination of O antigen and H antigen defines the serotype of an *E. coli*. Some specific *E. coli* serogroups are associated reproducibly with certain clinical syndromes, but it is not the serologic antigens themselves that confer virulence. Rather, the serotypes are served as identifiable chromosomal markers that correlate with specific virulent clones (58). Several *E. coli* pathotypes have been identified, including enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteroaggressive *E. coli*, and enteroinvasive *E. coli* (17).

E. coli O157:H7 which belongs to enterohemorrhagic *E. coli*, was first reported in the United States in 1983. It was associated with a multi-state outbreak of hemorrhagic colitis caused by the consumption of undercooked ground beef products (48). Since then, cattle have been identified as a principal reservoir of *E. coli* O157:H7 (40). As much as 28% of cattle fecal samples were positive for *E. coli* O157:H7 (20). Accordingly, contaminated ground beef has been identified as the source of infections in 48 out of 196 *E. coli* O157:H7 outbreaks documented in the United States between 1982 and 1998 (40). Most recently, the United States government ordered the second largest recall of beef in the U.S. history (approx. 19 million pounds of beef were recalled from 21 states) after at least 16 people infected from eating *E. coli* O157:H7 contaminated beef (4). In addition, a variety of other foods, such as apple cider, milk, cheese, yogurt, lettuce, water and sprouts have been involved in *E. coli* O157:H7 outbreaks.

The pathogenicity mechanism of *E. coli* O157:H7 has not been fully understood. Mainly, the pathogenicity of *E. coli* O157:H7 is determined by pathogenetic factors encoded by mobile genetic elements. The most important factors are the phage-encoded Shiga toxins (stx) elements (26, 27, 61) which are encoded for stx1 and stx2. Besides Shiga toxins, *E. coli* O157:H7 usually carries a large plasmid. On this plasmid, there are different virulent factors, such as the enterohemolysin E-Hly, the catalase-peroxidase KatP (10, 54), etc. The spectrum of *E. coli* O157:H7 infection includes diarrhea, hemorrhagic colitis, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura and death.

Most strains of *E. coli* O157:H7 also possess several characteristics uncommon to most other *E. coli*: inability to grow well, if at all, at temperatures $\geq 44.5^{\circ}\text{C}$ in *E. coli* (EC) broth, inability to ferment sorbitol within 24 hours, inability to produce β -glucuronidase (i.e. inability to hydrolyze 4-methylumbelliferyl-D-glucuronide, MUG)(32). Unlike most foodborne pathogens, many strains of *E. coli* O157:H7 are unusually tolerant to acidic environments(31, 38, 49).

Acid tolerance of *E. coli* O157:H7

The minimum pH for *E. coli* O157:H7 growth is 4.0-4.5, but is dependent upon the interaction of pH with other growth factors. It can survive in pH 2.0-2.5 gastric juice for at least 2 h because of common, as well as unique, strategies(22, 25). At pH higher than 3.0, it can survive for longer time. For example, apple cider which is acidic food and was considered free of pathogens became the vehicle of *E. coli* O157:H7 infection in a

food-borne outbreak(8). This high acid resistance ability may be related with the low infectious dose of *E. coli* O157:H7 (10-100 CFU) (37).

The ability of *E. coli* O157:H7 to survive in acid condition plays an important role in food and water borne diseases because of the acidic condition of stomach and intestine. Studies using acetic, citric, or lactic acid at concentrations up to 1.5% as organic acid sprays on beef revealed that *E. coli* O157:H7 populations were not appreciably affected by any of the treatments (9). *E. coli* O157:H7, when inoculated at high populations, survived fermentation, drying, and storage of fermented sausage (pH 4.5) for up to 2 months at 4°C (11, 23), mayonnaise (pH 3.6 to 3.9) for 5 to 7 weeks at 5°C and for 1 to 3 weeks at 20°C (28), and in apple cider (pH 3.6 to 4.0) for up to 42 days at 5 or 25°C (49), respectively. Outbreaks of *E. coli* O157:H7 infection have been directly associated with consumption of contaminated dry salami and apple cider. Several studies have described how enteric microorganisms cope with this form of environmental stress and referred to the acid survival systems as the acid tolerance response, acid resistance, and acid habituation (25, 36, 41, 45). Direct comparison of acid survival results among various groups (and microorganisms) has been difficult due to the use of complex versus minimal media, log-phase versus stationary-phase cells, and acid-challenge at various pH. Commonly higher level of acid resistance ability was found at the stationary phase cell for both gram-negative and gram-positive bacteria (7, 15, 21, 25).

Three systems in *E. coli* O157:H7 are reported that are involved in acid tolerance, including an acid-induced oxidative, an acid-induced arginine-dependent, and a glutamate-dependent system (47). The oxidative pathway is regulated by alternative sigma-transcription factor (*rpoS*) which is induced in cells grown on complex media as

they enter stationary growth phase(13, 25, 47). Once the oxidative system is active, the way in which it protects cells during acid-challenge is not clear. The *rpoS*-mediated system is not operative in fermentatively metabolizing cells (grown in complex medium containing glucose). The other two systems are activated in cells under fermentative growth condition, which require presence of amino acids during acid-challenge (6). The arginine decarboxylase system involves an arginine decarboxylase, *adiA* and an inner membrane antiporter which removes the decarboxylated product from the cytoplasm (13). The glutamate decarboxylase system involves a set of inducible genes encoding two nearly identical isoforms of glutamate decarboxylase (*gadA* and *gadB*) and an antiporter (*gadC*) that exchanges external glutamate for the intracellular decarboxylation product gamma-aminobutyric acid (13). The glutamate decarboxylase pathway is also induced at somewhat reduced levels during aerobic growth as cultures enter stationary growth phase.

Salmonella

Salmonellae are facultative anaerobic Gram-negative rods and belong to *Enterobacteriaceae* family. The organisms grow optically at 37 °C and catabolize D-glucose and other carbohydrates with the production of acid and gas. *Salmonellae* are oxidase negative and catalase positive, can grow on citrate as the sole carbon source, generally produce hydrogen sulfide, decarboxylate lysine and ornithine, and do not hydrolyze urea (5). Following the Kauffmann-White scheme, more than 2200 serovars have been recognized, whereas according to chromosomal DNA hybridization experiments and multilocus enzyme electrophoresis (MLEE), *Salmonella* can only be classified into eight subspecies- I, II, IIIa, IIIb, IV, V, VI, and VII(19, 33, 34). Species V

has been designated as *Salmonella bongori* which is rarely associated with human infections. *Salmonella enterica* comprises the remaining subspecies, such as *S. enterica* serovar Typhimurium, Enteritidis and Typhi.

Salmonella serotypes are closely related with pathogenicity and antibiotic-resistance. Different serotypes vary widely in niche and intrinsic pathogenicity. Most *Salmonella* virulence factors are clustered on chromosome referred as *Salmonella* pathogenicity islands (55). At least five *Salmonella* pathogenicity islands have been identified to date (53) which encode different secretion systems required by pathogenesis. The top four most commonly isolated serotypes from human infections were Typhimurium, Enteritis, Newport, and Heidelberg. These four serotypes were also the most frequently isolated serotypes from food samples (3, 14).

As one of the most common foodborne pathogens, nontyphoidal *Salmonella* have a wide range of hosts and are strongly associated with food animal products. Ninety-five percent of nontyphoidal *Salmonella* infection cases are food-borne and salmonellosis accounts for more than 30% of deaths resulted from food-borne diseases in the United States (39). Notable recent outbreaks of *Salmonella* infections have been linked to eggs, cheese, ice cream premix, a variety of fresh sprouts, juice, cantaloupes, and other fresh vegetables (42, 43, 50, 52). The symptoms of salmonellosis include diarrhea, nausea, vomiting, fever and abdominal cramps. When the infection spreads beyond the intestinal tract, appropriate antimicrobials therapy is necessary and can be lifesaving. However, an increasing number of *Salmonella* have developed resistance to many of the antimicrobials commonly used for treatment, which can have a major impact on human and animal health.

Antibiotic-resistant *Salmonella*

Increasing antimicrobial resistance has become one of the most common concerns of foodborne *Salmonellae*. As more than 90% salmonellosis are foodborne, antibiotic-resistant *Salmonella* in food-producing animals has become a focus of the debate on antibiotic use in food animals. Research has shown that the frequency of antibiotic-resistant bacteria is closely related with the volume of antibiotics used (51, 59). There are 32 different antibiotics used in conventional broiler feeds for growth promotion, disease prevention and treatment without veterinary prescription. Each year, 17.62 million pounds of antibiotics are used to prevent and treat animal diseases and 2.8 million pounds are used for animal growth promotion (12).

Use of antibiotics in animals selects the growth and spread of resistant strains, which will accumulate in the farm environment and may become the dominant strain under selective pressure. This will limit the therapeutic options available to veterinarians and physicians in treatment of bacterial infections. For example, *Salmonella* Typhimurium strain DT 104 which is resistant to five antibiotics: ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (ACSSuT), has been detected worldwide, including Europe and the United States (29, 35, 46, 60). In 1999, the data from National Antimicrobial Resistance Monitoring Systems (NARMS) showed that 28% of the 362 *S. Typhimurium* isolates had the ACSSuT resistant pattern (2).

Table 1.2. Most frequently reported *Salmonella* serotypes recovered from humans in the United States in 2002 (14)

Rank	Serotype	Number of isolates	Percentage (%)
1	Typhimurium	6999	22.1
2	Enteritidis	5614	17.7
3	Newport	3158	10.0
4	Heidelberg	1884	5.9
5	Javiana	1067	3.4
6	Montevideo	626	2.0
7	Oranienburg	595	1.9
8	Muenchen	583	1.8
9	Thompson	514	1.6
10	Saintpaul	469	1.5
11	Java	466	1.5
12	Infantis	440	1.4
13	Braenderup	388	1.2
14	Agona	370	1.2
15	Typhi	343	1.1
16	Mississippi	336	1.1
17	Berta	330	1.0
18	Poona	330	1.0
19	Hadar	307	1.0
20	Bareilly	205	0.6
Sub Total		25024	79.0
Total		31675	100.0

Detection and identification of foodborne pathogens

Detection of bacterial pathogens in food products is important to control and prevent foodborne illnesses. There are mainly four groups of detection methods available for foodborne pathogens: conventional, immunological, molecular, and biosensors method. Food laboratories rely mainly on microbiological culture, enrichment, isolation, and phenotypic analysis, followed by serological confirmation in a process lasting several days. Clinical laboratories with fewer budgetary constraints have a range of more rapid but costly methods available. In most instances detection methods for use with foods require greater specifications of specificity and sensitivity at lower cost than clinical applications because in disease, the human host selected for sampling carries a high likelihood of being positive, the patient provides enriched cultures of pathogens in batches of a single sample type (feces), and false positive results are unlikely. Food analysis must contend with a) a great variety of matrices, b) low level uneven contamination, c) in-line continuous processing, and d) strict cost constraints.

a. Conventional methods

The detail of this group of methods is provided in the USDA/FSIS, and FDA microbiology laboratory guidebooks (1, 5). Because of low pathogen concentration in food samples, non-selective and selective enrichment steps are applied to increase the pathogen concentration to a detectable level and followed by isolation methods with selective and/or differential media. Finally, colonies of presumptive positive bacteria will be confirmed using biochemical tests or serological tests. It may take 3-5 days to detect *E. coli* O157:H7 and *Salmonella* from food samples. The advantages of these methods

include that bacteria strains are isolated and may be used for further analysis, and only living bacteria in food samples are identified.

b. Immunological methods

Immunological detection methods, such as ELISA, immunofluorescent antibody (IFA) techniques, and radioimmunoassay, are based on the specific binding of antibodies to antigens. Compared with the conventional methods, the immunological methods are faster, taking only 10-28 h to have the results. The disadvantages include: (i) because they are based on the antigen and antibody reaction, false positive results may occur; (ii) they are not able to differentiate the dead and living cells since the antigens are present on both dead and living cells; and (iii) there are no isolates available for further studies. Due to these disadvantages, the immunological methods are usually used as a screening method. Immunological techniques can also be used in sample preparation for further bacterial identification, such as immunomagnetic separation(24, 44, 57).

c. Biosensor based methods

Biosensor is composed of a biological recognition element (such as an enzyme, antibody, receptor, or nucleic acid) coupled to a chemical or physical transducer. Today, there are four major types of transducers: electrochemical (electrodes), mass (piezoelectric crystals or surface acoustic wave device), optical (optrodes) and thermal (thermistors or heat-sensitive sensors) transducers. The biological recognition element can specifically identify and bind the target analytes, it can form a signal passing through

the transducer. But for the non-target analytes, they can not bind to the biological recognition element, so there are no signals from the non-target analytes in theory.

By using different biological recognition elements and different transducers, many kinds of biosensors can be developed. Currently, 90% biosensors used on pathogen detection depend on immunological reactions. Immunological based biosensors, immunosensors, are composed of an antigen recognition element (antibody) and a chemical or physical transducer. In such a biosensor, the antibodies can bind specific antigens from food products, and the transducer can translate this signal to a detectable signal. The specificity of antibodies used in biosensors determines the specificity of the biosensor. Usually mono-clone antibodies are used in biosensors to have a high specificity for pathogen detection. There are many reports claiming that their methods are rapid (detection time less than 1h), sensitive (detect 1 cell in food sample) and specific (specifically detect the target pathogen.). But many of the studies did not report the time spent on samples and biosensors preparation. When the specificity of biosensors is determined, organisms closely related to the target pathogen were often not included.

d. Molecular detection methods

Molecular detection methods based on specific composition of the pathogens' genetic materials include DNA hybridization and DNA amplification, such as PCR method. Indeed, molecular methods have become popular and reliable techniques for differentiating diarrheagenic strains from nonpathogenic strains. Currently, *E. coli* O157:H7 and *Salmonella* specific genes such as virulent genes *stx1*, *stx2*, invasion genes *invA*, are the targets for this group of methods. As the sequences of the complete

genomes of *E. coli* O157:H7 and *Salmonella* Typhimurium have become available, more DNA sequences specific for the pathogens will be identified and used for detecting the pathogens. For the last several years, real-time quantitative PCR assays have been introduced to clinical and food laboratories. By analyzing the fluorescent signals collected by a computer, pathogenic organisms and the number of these organisms in food samples can be determined simultaneously.

Although significant progresses have been made in developing rapid, sensitive, and specific detection methods, many challenges in the detection of microbial pathogens in food remain. Perfect tests with specific criteria listed in Table 1.3 are still not available.

Project description

My Ph.D. research focused on detection and characterization of *E. coli* O157:H7 and *Salmonella* in food. We identified cell density-dependent survival phenotype of *E. coli* O157:H7 under acidic conditions, developed food sample preparation procedures for PCR detection of *E. coli* O157:H7, improved the methodology in isolation and identification of *Salmonella* from meat products, characterized antimicrobial resistance of *Salmonella* from retail chickens raised under conventional and organic productions.

Table 1.3. Summary of an ideal pathogens detection method used in food samples(16)

Sensitivity	Ability to detect single bacteria in a reasonably sample volume.
Species selectivity	Ability to distinguish individual bacterial species in the presence of other microorganisms or cells.
Strain selectivity	Ability to distinguish an individual bacterial strain from other strains of the same species.
Assay time	5-10 min for a single test, useful for field testing.
Assay protocol	No reagent addition needed
Measurement	Direct, without pre-enrichment
Format	Highly automated format (“single button device”)
Operator	No skills required using the assay.
Viable cell count	Should discriminate live and dead cells
Size	Compact, portable, hand-held, design for field use
Strain isolation	The pathogen strains should be available for further study.
Cost	It should be very cheap for negative samples

In chapter two, the cell density-dependent acid sensitivity phenotypes of *E. coli* strains K-12 and O157:H7 were examined during stationary growth phase. High cell density suspensions (2×10^9 CFU/ml) survived acid-challenge very poorly ($\leq 0.004\%$ survival) in Luria-Bertani broth (pH 2.5, 37 °C, 2 h). The same cultures, when subjected to an identical acid-challenge at lower cell density (10^6 CFU/ml), showed higher survival ($\geq 15\%$). The cell density-dependent acid sensitive phenotype of *E. coli* was analyzed with reference to three possible mechanisms of acid resistance.

There was no evidence of any diffusible substance released from dead cells, which could influence the cell density-dependent acid-survival phenotype. Nor was there evidence for the involvement of the alternative sigma-transcription factor (*rpoS*). Instead, cell-density dependent acid-survival phenotype was associated with induction of glutamate- and arginine-decarboxylase acid-survival pathways and concomitant availability of glutamate and arginine during acid-challenge. The data revealed that during acid-challenge at high cell density, the limited availability of glutamate and/or arginine creates an illusion of cell density-dependent acid sensitive phenotype.

In chapter three, an improved, rapid and sensitive sample preparation method for PCR-based detection of *E. coli* O157:H7 in ground beef was developed, fresh ground beef samples were experimentally inoculated with varying concentrations of *E. coli* O157:H7. PCR inhibitors were removed and bacterial cells were concentrated by filtration, centrifugation, and lysed using enzymatic digestion and successive freeze/thaw cycles. DNA was purified and concentrated via phenol/chloroform extraction and the Shiga toxin 1 gene (*stxI*) was amplified using PCR to evaluate the sample preparation method. Without prior enrichment of cells in broth media, the detection limit was 10^3

CFU/g beef. When a 6-h enrichment step was incorporated, the detection limit was 1 CFU/g beef. Total time required from beginning to end of the procedure is 12 h. The sample preparation method developed enabled substantially improved sensitivity in the PCR-based detection of *E. coli* O157:H7 in ground beef, as compared to previous reports. Superb sensitivity, coupled with quick turn-around time, relative ease of use, and cost-effectiveness, makes it a useful method for detecting *E. coli* O157:H7 in ground beef.

In chapter four, an improved procedure based on the method described in the USDA/Food Safety Inspection Service (FSIS) Microbiological Laboratory Guidebook was developed to isolate *Salmonella* from poultry products. The modified method was evaluated using the standard procedures of the FDA/Bacteriological Analytical Manual (BAM) and USDA/FSIS Microbiological Laboratory Guidebook. Greater than 95% of the presumptive *Salmonella* colonies isolated using the modified method from chicken samples were confirmed as *Salmonella* by PCR/ API 20E tests. Its high specificity significantly reduced the time for isolation and identification of *Salmonella* from poultry products (48 h compared to 3-5 days); thus, the costs were reduced. Since the method has a high specificity, *Salmonella* isolates may be further subtyped by serotyping and/or pulsed-field gel electrophoresis (PFGE) without biochemical confirmation. Using this method, *Salmonella* were recovered from 29 of 44 (66%) retail organically produced chicken samples. The widespread occurrence of *Salmonella* in organic chicken samples emphasizes the need for implementation of effective control measures of microbial contaminations in organic poultry products.

In chapter 5, *Salmonella* in retail organic and conventional chicken samples were isolated and characterized by serotyping, antimicrobial-susceptibility testing, and pulse-

field gel electrophoresis (PFGE). Among 198 organic chicken samples, 121 (61%) were *Salmonella* positive, compared to 27 (44%) out of 61 conventional chicken samples. Kentucky, Heidelberg, and Typhimurium were top three serotypes identified and accounted for more than 60% of all *Salmonella* isolates from chicken samples. There were 37 PFGE patterns identified among isolates from 148 chicken samples, of which 14 patterns were found only in conventional chicken samples, 18 patterns were found only in organic chicken samples, and 5 patterns were shared by both conventional and organic chicken samples. Antimicrobial-susceptibility testing results showed that antimicrobial resistance in *Salmonella* was associated with serotypes. All *S. Typhimurium* positive conventional chicken samples contained isolates that were resistant to at least one antimicrobial compared to only 21% of organic chicken samples. The similar trend was found among other serotypes. Our results indicated that organic chicken samples were frequently contaminated with *Salmonella*, and that *Salmonella* from organic chicken were more susceptible to antimicrobials commonly used in human and veterinary medicine.

REFERENCES

1. FDA Bacteriological analytical manual. [Online.] <http://www.foodinfonet.com/publication/fdaBAM.htm>. Accessed April 12, 2004.
2. NARMS 1999 Annual Report. CDC. [Online.] <http://www.cdc.gov/ncidod/dbmd/narms/annual/1999/html/99sum.htm>. Accessed April 12, 2004.
3. Preliminary FoodNet data on the incidence of foodborne illnesses--selected sites, United States, 2000. *MMWR Morb Mortal Wkly Rep* **50**:241-6.
4. Recall Notification Report. Food Safety and Inspection Service, United States Department of Agriculture, Washington, DC. [Online.] <http://www.fsis.usda.gov/OA/recalls/rnrfiles/rnr055-2002.htm>. Accessed April 12, 2004.

5. USDA/FSIS Microbiology laboratory guidebook. [Online.] <http://www.fsis.usda.gov/OPHS/microlab/mlgbook.htm>. Accessed April 12, 2004.
6. **Bearson, S., B. Bearson, and J. W. Foster.** 1997. Acid stress responses in enterobacteria. *FEMS Microbiol Lett* **147**:173-80.
7. **Benjamin, M. M., and A. R. Datta.** 1995. Acid tolerance of enterohemorrhagic *Escherichia coli*. *Appl Environ Microbiol* **61**:1669-72.
8. **Besser, R. E., S. M. Lett, J. T. Weber, M. P. Doyle, T. J. Barrett, J. G. Wells, and P. M. Griffin.** 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. *Jama* **269**:2217-20.
9. **Bolton, D. J., T. Catarama, C. Byrne, J. J. Sheridan, D. A. McDowell, and I. S. Blair.** 2002. The ineffectiveness of organic acids, freezing and pulsed electric fields to control *Escherichia coli* O157:H7 in beef burgers. *Lett Appl Microbiol* **34**:139-43.
10. **Bruner, W., H. Schmidt, and H. Karch.** 1996. KatP, a novel catalase-peroxidase encoded by the large plasmid of enterohaemorrhagic *Escherichia coli* O157:H7. *Microbiol* **142** (Pt 11):3305-15.
11. **Calicioglu, M., N. G. Faith, D. R. Buege, and J. B. Luchansky.** 2002. Viability of *Escherichia coli* O157:H7 during manufacturing and storage of a fermented, semidry soudjouk-style sausage. *J Food Prot* **65**:1541-4.
12. **Carnevale, R.** 2001. Antibiotic resistance and food producing animals-Views of the industry. [Online.] <http://www.ahi.org>. Accessed July 1st, 2002.
13. **Castanie-Cornet, M. P., T. A. Penfound, D. Smith, J. F. Elliott, and J. W. Foster.** 1999. Control of acid resistance in *Escherichia coli*. *J Bacteriol* **181**:3525-35.
14. **CDC.** 2003. *Salmonella* Surveillance Summary, 2002. US Department of Health and Human Services.
15. **Datta, A. R., and M. M. Benjamin.** 1997. Factors controlling acid tolerance of *Listeria monocytogenes*: effects of nisin and other ionophores. *Appl Environ Microbiol* **63**:4123-6.
16. **I. Dmitri, A. Plamen, W. Ebtisam, S. Stephen.** 2000. Application of electrochemical biosensors for detection of food pathogenic bacteria. *Electroanalysis* **12**:317-325.

17. **Donnenberg, M. S., and T. S. Whittam.** 2001. Pathogenesis and evolution of virulence in enteropathogenic and enterohemorrhagic *Escherichia coli*. *J Clin Invest* **107**:539-48.
18. **Edwards, P. R., and W. H. Ewing.** 1972. Identification of *Enterobacteriaceae*. Burgess Publishing Co., Minneapolis, Minn.
19. **Eiguer, T., and M. I. Caffer.** 1988. [*Salmonella* taxonomy]. *Rev Argent Microbiol* **20**:151-4.
20. **Elder, R. O., J. E. Keen, G. R. Siragusa, G. A. Barkocy-Gallagher, M. Koohmaraie, and W. W. Laegreid.** 2000. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proceedings of the National Academy of Sciences of the United States of America* **97**:2999-3003.
21. **Foster, J. W.** 1995. Low pH adaptation and the acid tolerance response of *Salmonella* Typhimurium. *Crit Rev Microbiol* **21**:215-37.
22. **Foster, J. W.** 1999. When protons attack: microbial strategies of acid adaptation. *Curr Opin Microbiol* **2**:170-4.
23. **Glass, K. A., J. M. Loeffelholz, J. P. Ford, and M. P. Doyle.** 1992. Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. *Appl Environ Microbiol* **58**:2513-6.
24. **Gooding, C. M., and P. V. Choudary.** 1997. Rapid and sensitive immunomagnetic separation-polymerase chain reaction method for the detection of *Escherichia coli* O157:H7 in raw milk and ice-cream. *J Dairy Res* **64**:87-93.
25. **Gorden, J., and P. L. Small.** 1993. Acid resistance in enteric bacteria. *Infect Immun* **61**:364-7.
26. **Griffin, P. M., S. M. Ostroff, R. V. Tauxe, K. D. Greene, J. G. Wells, J. H. Lewis, and P. A. Blake.** 1988. Illnesses associated with *Escherichia coli* O157:H7 infections. A broad clinical spectrum. *Ann Intern Med* **109**:705-12.
27. **Griffin, P. M., and R. V. Tauxe.** 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol Rev* **13**:60-98.
28. **Hathcox, A. K., L. R. Beuchat, and M. P. Doyle.** 1995. Death of enterohemorrhagic *Escherichia coli* O157:H7 in real mayonnaise and reduced-calorie mayonnaise dressing as influenced by initial population and storage temperature. *Appl Environ Microbiol* **61**:4172-7.

29. **Helms, M., P. Vastrup, P. Gerner-Smidt, and K. Molbak.** 2002. Excess mortality associated with antimicrobial drug-resistant *Salmonella* Typhimurium. *Emerg Infect Dis* **8**:490-5.
30. **Jay, J. M.** 2000. *Modern Food Microbiology*, sixth edition. Aspen Publishers, Inc, Gaithersburg, MD.
31. **Jordan, K. N., L. Oxford, and C. P. O'Byrne.** 1999. Survival of low-pH stress by *Escherichia coli* O157:H7: correlation between alterations in the cell envelope and increased acid tolerance. *Appl Environ Microbiol* **65**:3048-55.
32. **Kim, J., J. Nietfeldt, J. Ju, J. Wise, N. Fegan, P. Desmarchelier, and A. K. Benson.** 2001. Ancestral divergence, genome diversification, and phylogeographic variation in subpopulations of sorbitol-negative, beta-glucuronidase-negative enterohemorrhagic *Escherichia coli* O157. *J Bacteriol* **183**:6885-97.
33. **Le Minor, L., M. Y. Popoff, B. Laurent, and D. Hermant.** 1986. [Characterization of a 7th subspecies of *Salmonella*: *S. Choleraesuis* subsp. indica subsp. nov.]. *Ann Inst Pasteur Microbiol* **137B**:211-7.
34. **Le Minor, L., M. Veron, and M. Popoff.** 1982. [The taxonomy of *Salmonella*]. *Ann Microbiol (Paris)* **133**:223-43.
35. **Liebana, E., L. Garcia-Migura, C. Clouting, F. A. Clifton-Hadley, E. Lindsay, E. J. Threlfall, S. W. McDowell, and R. H. Davies.** 2002. Multiple genetic typing of *Salmonella enterica* serotype typhimurium isolates of different phage types (DT104, U302, DT204b, and DT49) from animals and humans in England, Wales, and Northern Ireland. *J Clin Microbiol* **40**:4450-6.
36. **Lin, J., I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster.** 1995. Comparative analysis of extreme acid survival in *Salmonella* Typhimurium, *Shigella flexneri*, and *Escherichia coli*. *J Bacteriol* **177**:4097-104.
37. **Lin, J., M. P. Smith, K. C. Chapin, H. S. Baik, G. N. Bennett, and J. W. Foster.** 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. *Appl Environ Microbiol* **62**:3094-100.
38. **Marques, P. A., D. Worcman-Barninka, S. C. Lannes, and M. Landgraf.** 2001. Acid tolerance and survival of *Escherichia coli* O157:H7 inoculated in fruit pulps stored under refrigeration. *J Food Prot* **64**:1674-8.
39. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. *Emerg Infect Dis* **5**:607-25.

40. **Meng, J., M. P. Doyle, T. Zhao, and S. Zhao.** 2001. Enterohemorrhagic *Escherichia coli*. In Food microbiology: fundamentals and frontiers. American Society of Microbiology Press, Washington, DC.
41. **O'Hara, G. W., and A. R. Glenn.** 1994. The adaptive acid tolerance response in root nodule bacteria and *Escherichia coli*. Arch Microbiol **161**:286-92.
42. **O'Ryan, M.** 1996. An outbreak of *Salmonella* infection from ice cream. N Engl J Med **335**:824; author reply 824-5.
43. **Panico, M. G., F. Primiano, F. Nappi, and F. Attena.** 1999. An outbreak of *Salmonella* Enteritidis food poisoning from a commercially produced cheese. Euro Surveill **4**:47-48.
44. **Parham, N., J. Spencer, D. Taylor, H. Ternent, G. Innocent, D. Mellor, M. Roberts, and A. Williams.** 2003. An adapted ImmunoMagnetic cell separation method for use in quantification of *Escherichia coli* O157:H7 from bovine faeces. J Microbiol Methods **53**:1-9.
45. **Raja, N., M. Goodson, W. C. Chui, D. G. Smith, and R. J. Rowbury.** 1991. Habituation to acid in *Escherichia coli*: conditions for habituation and its effects on plasmid transfer. J Appl Bacteriol **70**:59-65.
46. **Rajashekara, G., E. Haverly, D. A. Halvorson, K. E. Ferris, D. C. Lauer, and K. V. Nagaraja.** 2000. Multidrug-resistant *Salmonella* Typhimurium DT104 in poultry. J Food Prot **63**:155-61.
47. **Richard, H. T., and J. W. Foster.** 2003. Acid resistance in *Escherichia coli*. Adv Appl Microbiol **52**:167-86.
48. **Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen.** 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. New England Journal of Medicine **308**:681-685.
49. **Ryu, J. H., and L. R. Beuchat.** 1998. Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. Int J Food Microbiol **45**:185-93.
50. **Sagoo, S. K., C. L. Little, L. Ward, I. A. Gillespie, and R. T. Mitchell.** 2003. Microbiological study of ready-to-eat salad vegetables from retail establishments uncovers a national outbreak of salmonellosis. J Food Prot **66**:403-9.
51. **Salyers, A. A.** 2002. An overview of the genetic basis of antibiotic resistance in bacteria and its implications for agriculture. Anim Biotechnol **13**:1-5.

52. **Sarna, M., G. Dowse, G. Evans, and C. Guest.** 2002. An outbreak of *Salmonella* Typhimurium PT135 gastroenteritis associated with a minimally cooked dessert containing raw eggs. *Commun Dis Intell* **26**:32-7.
53. **Schmidt, H., and M. Hensel.** 2004. Pathogenicity islands in bacterial pathogenesis. *Clin Microbiol Rev* **17**:14-56.
54. **Schmidt, H., C. Kernbach, and H. Karch.** 1996. Analysis of the EHEC hly operon and its location in the physical map of the large plasmid of enterohaemorrhagic *Escherichia coli* O157:h7. *Microbiology* **142 (Pt 4)**:907-14.
55. **Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden.** 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella* Typhimurium. *Proc Natl Acad Sci U S A* **93**:2593-7.
56. **Tauxe, R. V.** 2002. Emerging foodborne pathogens. *Int J Food Microbiol* **78**:31-41.
57. **Tomoyasu, T.** 1998. Improvement of the immunomagnetic separation method selective for *Escherichia coli* O157 strains. *Appl Environ Microbiol* **64**:376-82.
58. **Whittam, T. S., M. L. Wolfe, I. K. Wachsmuth, F. Orskov, I. Orskov, and R. A. Wilson.** 1993. Clonal relationships among *Escherichia coli* strains that cause hemorrhagic colitis and infantile diarrhea. *Infect. Immun.* **61**:1619-1629.
59. **Witte, W.** 1998. Medical consequences of antibiotic use in agriculture. *Science* **279**:996-7.
60. **Zhao, T., M. P. Doyle, P. J. Fedorka-Cray, P. Zhao, and S. Ladely.** 2002. Occurrence of *Salmonella enterica* serotype typhimurium DT104A in retail ground beef. *J Food Prot* **65**:403-7.
61. **Zueva, N. N., T. A. Popova, A. Sypchenko, and L. I. Shishkina.** 2000. [The epidemiology of acute intestinal infections complicated by the development of the hemolytic-uremic syndrome in Tula Province]. *Zh Mikrobiol Epidemiol Immunobiol*:105-7.

Chapter 2 Availability of Glutamate and Arginine during Acid- challenge Determines Cell Density-dependent Survival Phenotype of *Escherichia coli* Strains

ABSTRACT

The cell density-dependent acid sensitivity phenotypes of *E. coli* strains K-12 and O157:H7 were examined during stationary growth phase. High cell density suspensions (10^9 CFU/ml) survived acid-challenge very poorly ($\leq 0.004\%$ survival) in Luria-Bertani broth (pH 2.5, 37°C, 2 h). The same cultures, when subjected to an identical acid-challenge at lower cell density (10^6 CFU/ml), showed higher survival ($\geq 15\%$). The cell density-dependent acid sensitive phenotype of *E. coli* was analyzed with reference to three possible mechanisms of acid resistance. There was no evidence of any diffusible substance released from dead cells, which could influence the cell density-dependent acid-survival phenotype. Nor was there evidence for the involvement of the alternative sigma-transcription factor (*rpoS*). Instead, cell-density dependent acid-survival phenotype was associated with induction of glutamate- and arginine-decarboxylase acid-survival pathways and concomitant availability of glutamate and arginine during acid-challenge. The data revealed that during acid-challenge at high cell density, the limited availability of glutamate and/or arginine creates an illusion of cell density-dependent acid sensitive phenotype.

INTRODUCTION

During their pathogenic and commensal lifestyle, an acidic environment is a common stress encountered by enteric bacteria such as *Salmonella* Typhimurium and *E. coli*. In order to survive such potentially lethal acid conditions, *S. Typhimurium* and *E. coli* have evolved common, as well as unique, strategies (10, 14). Several studies have described how enteric microorganisms cope with this form of environmental stress and referred to the acid survival systems as the acid tolerance response, acid resistance, and acid habituation (14, 17, 18, 20). Direct comparison of acid survival results among various groups (and microorganisms) has been difficult due to the use of complex versus minimal media, log-phase versus stationary-phase cells, and acid-challenge at various pHs (11, 14, 24). To add further complexity to the analysis, there appear to be substances secreted by cells that apparently influence acid sensitivity (22, 23). Among various extracellular components which have been reported to influence acid tolerance, synthesis of some of the diffusible components from enterohemorrhagic *E. coli* (EHEC) strains appeared to be dependent on the synthesis of an alternative sigma-transcription factor, *rpoS* (7). All the EHEC strains and *Shigella* spp. examined reportedly synthesized the diffusible substances irrespective of their serotype or their ability to synthesize Shiga-like toxins. These substances were postulated to regulate cell density-dependent acid survival responses in a manner similar to *N*-acyl-L-homoserine lactones (AHLs) (7, 16).

Few studies have directly examined the effect of cell density, acid pH, and growth conditions on survival of pathogenic *E. coli* strains. Earlier studies examined this relationship without considering the possibility that exposure to different growth conditions might influence the ultimate outcome of acid-challenge (1, 2, 13). Recent

analysis of the molecular aspects of acid tolerance pathways in *E. coli* has opened up new strategies capable of dissecting cell density-dependent acid sensitivity phenotype (6, 12). Our aim was to identify putative diffusible substances involved in cell density-dependent acid sensitivity in *E. coli* with the knowledge that multiple acid resistance systems may be involved.

Three pathways have been identified which enable *E. coli* to survive acid-challenge. One is glucose repressible, oxidative pathway regulated by alternative sigma-transcription factor (*rpoS*) which is induced in cells grown on complex media as they enter stationary growth phase. Once the oxidative system is active, the way in which it protects cells during acid-challenge remains a mystery. The *rpoS*-mediated system is not operative in fermentatively metabolizing cells (grown in complex medium containing glucose). Two other acid resistant systems are activated in cells under this growth condition, which require presence of amino acids during acid-challenge (4). These two systems are known as the glutamate decarboxylase system (*gadABC* operon) (15) and the arginine decarboxylase system (*adiA*) (17). The glutamate decarboxylase pathway is also induced at somewhat reduced levels during aerobic growth as cultures enter stationary growth phase.

In this study we have addressed the issue of diffusible substances released from *E. coli* strains during acid-challenge (7, 23). Our data confirmed cell density-dependent acid survival in *E. coli*. However, no direct evidence for the occurrence of diffusible substances, which could induce cell density-dependent acid sensitivity, was obtained. On the contrary, the data indicated that the absence of certain amino acids or their limited

availability during acid-challenge gives a phenotype of cell density-dependent acid sensitivity.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The *E. coli* and *S. Typhimurium* strains used in this study are listed in Table 2.1. Cultures were streaked on Luria Bertani (LB) agar plates from freezer stocks and a single colony was inoculated in LB broth. Cultures were inoculated in 10 ml LB broth in a 125-ml flask, which was incubated on a shaker incubator at 37 °C, 230 rpm for 18 to 20 h (oxidative growth). For fermentative growth, cultures were started from a single colony in LB broth containing 0.4% glucose (adjusted to pH 5.0) as described by Lin *et al.*, (17). Briefly, 2.5 ml of broth was placed in a sterile tube (100 x 11 mm) which was placed at a 45° angle in a shaker incubator at 37 °C at 230 rpm. After incubation for 18-20 h, cultures had a pH range of 4.5 to 4.7.

Acid-challenge assays

Cultures grown for 18-20 h, were centrifuged and washed once in sterile saline and resuspended in saline at various cell densities. The washed cell suspensions were subjected to acid-challenge in either acidified LB broth (pH 2.5, acidified with HCl) or synthetic gastric juice (2, 5) for 2 h at 37 °C. Acid-challenge was performed in presence of glutamate or arginine at different concentrations. Cells were diluted in sterile phosphate-buffered saline before estimating the viable cell count.

Table 2.1. *E. coli* and *Salmonella* strains used in this study

Strain	Serotype or genotype ^a	Reference
<i>E. coli</i>		
MG 1655	K-12, wild-type	(21)
EK274	O157:H7 wild-type (ATCC 43895), Na ^r Rf ^r	(19)
EK275	EK274 <i>rpoS</i> ::pRR10 Amp ^r	(19)
EK484	EK274 <i>gadC</i> ::pRR10 Amp ^r	Price and Foster, unpublished data
EK489	EK274 <i>adiA</i> ::pRR10 Amp ^r	Price and Foster, unpublished data
<i>S. Typhimurium</i>	ATCC 14028s wild-type	(9)

^aAbbreviations for antibiotics: Na, nalidixic acid; Rf, rifampin; Amp, ampicillin.

To obtain “used” synthetic gastric juice, cell suspensions after acid-challenge in fresh gastric juice (using 10^9 CFU/ml) were centrifuged at $10,000 \times g$ and the supernatant was passed through a $0.2 \mu\text{m}$ nylon filter. The filtered sterilized synthetic gastric juice was referred to as “used” gastric juice and was either utilized immediately for acid-challenge assay or in some cases stored at -20°C until use.

RESULTS

Acid-challenge at various cell densities

Experiments were designed to examine the potential presence of factors associated with cell-to-cell communication, especially those which may be associated with cell density-dependent acid sensitivity. High (10^9 CFU/ml) and low (10^6 CFU/ml) cell density suspensions of *E. coli* O157:H7 and *E. coli* K-12 (MG 1665) were subjected to acid-challenge in an acidified LB medium (pH 2.5, 37°C , 2 h). In order to facilitate subsequent purification of diffusible substances, which may be released from dead cells (7, 23), experiments were also repeated in synthetic gastric juice under identical conditions. Both strains survived poorly under the test conditions of high cell density during acid-challenge. However, the same cell preparations were resistant to identical acid-challenge at lower cell densities (Figure 2.1). Next, we examined the properties of the acidic LB medium or synthetic gastric juice in which acid-challenge assays were performed. Cells challenged at both low and high cell density survived poorly in the “used” synthetic gastric juice (Table 2.2). The failure of low cell density population to survive in the “used” synthetic gastric juice was further examined to determine whether it

was due to substances released from the dying high cell density population during acid-challenge.

Table 2.2. Survival of high and low density cell suspensions during acid-challenge.

Strain	Cell density during acid-challenge (CFU/ml)	% Survival	
		Nature of acid-challenge	
		Fresh gastric juice pH 2.5, 37 °C, 2 h	“used” gastric juice ¹ pH 2.5, 37 °C, 2 h
<i>E. coli</i> K 12 (MG 1655)	2 x 10 ⁹	0.0037 ± 0.0002	0.0051 ± 0.00004
	2 x 10 ⁶	15.00 ± 6.01	0.0026 ± 0.0002
<i>E. coli</i> O157:H7 (EK274)	2 x 10 ⁹	0.0014 ± 0.00002	≤ 0.00001
	2 x 10 ⁶	25.5 ± 6.37	≤ 0.00001

¹Fresh gastric juice in which cells (10⁹ CFU / ml) were subjected to acid-challenge (37 °C, 2 h) was centrifuged, and supernatant was filter sterilized to obtain “used” gastric juice.

Acid-challenge in the presence of substances released from dead cells

In order to obtain large quantities of the putative factors at higher concentration, cell suspensions of very high cell density (ca. 10⁹ to 10¹⁰ CFU/ml) were subjected to acid-challenge in synthetic gastric juice. It was reasoned that killing large cell populations in small volumes would provide large quantities of crude diffusible

substances that might be responsible for cell density-dependent acid sensitivity. We continued to observe poor survival rates ($\leq 0.004\%$ survival) at cell densities as high as 10^{10} cells per ml of synthetic gastric juice (data not shown). After filter-sterilization, various dilutions of “used” synthetic gastric juice made in fresh synthetic gastric juice were tested for potency in acid-challenge assays using low cell density suspensions (Figure. 2.2). It was observed that irrespective of the initial cell concentration used to obtain the ‘used’ synthetic gastric juice (i.e., 10^8 CFU/ml or 10^{10} CFU/ml), only undiluted preparations were effective in providing acid-mediated killing of cells at low cell density. In contrast, addition of small quantities of fresh synthetic gastric juice were sufficient to restore acid survival (Figure. 2.2), indicating that it may be an absence of factor in the ‘used’ synthetic gastric juice that may be responsible for the low survival rate during acid-challenge.

Role of individual acid survival pathways

Of the three pathways by which *E. coli* cells survive acid-challenge (6), i.e., oxidative pathway (*rpoS* mediated), and two amino acid decarboxylase pathways [mediated by glutamate decarboxylase (*gadABC* operon) and arginine decarboxylase (*adiA*)], the glutamate decarboxylase pathway is expressed under both aerobic as well as fermentative growth conditions, while arginine decarboxylase is induced strictly under fermentative growth.

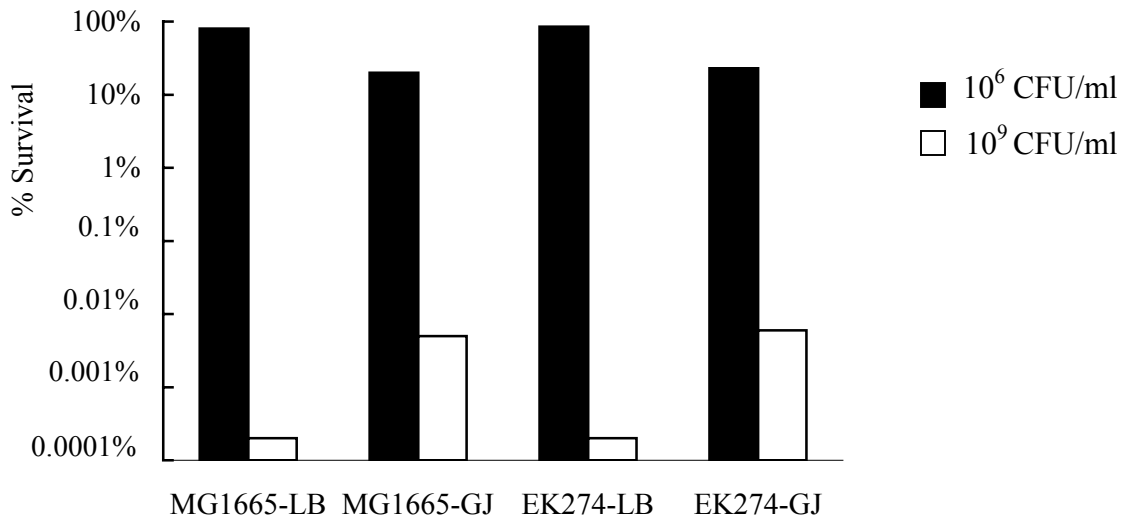


Figure 2.1. Effect of cell density during acid-challenge on survival of *E. coli* strains.

Strains MG1665 and EK274 were acid-challenged at low (10^6 CFU/ml, closed symbols) and high (10^9 CFU/ml, open symbols) cell densities in fresh synthetic gastric juice or acidified LB broth. Acid-challenge was performed at pH 2.5, 37 °C for 2 h.

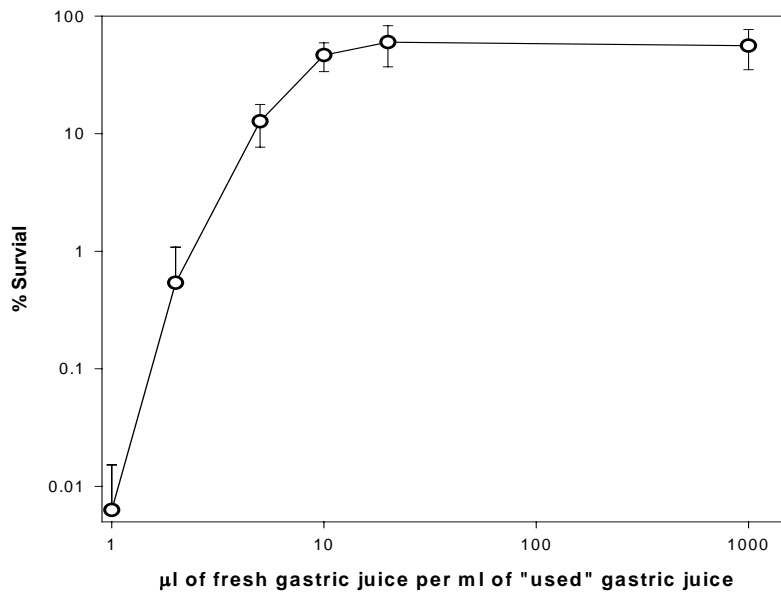


Figure 2.2. Survival of *E. coli* O157:H7 in “used” synthetic gastric juice supplemented with varying quantities of fresh synthetic gastric juice. Acid-challenge was performed at low cell density (10^6 CFU/ml). Error bars represent standard deviation.

Using mutants of each of the three acid survival pathways, it was determined whether cell density-dependent acid sensitivity is due to a limited availability of amino acid during acid-challenge.

a. Effect of *rpoS* on cell density-dependent acid sensitivity

The oxidative acid resistant pathway is fully expressed during the stationary growth phase of aerobically grown cells and requires the *rpoS* gene product, an alternative σ -factor for transcription. *E. coli* strains defective in *rpoS* were shown previously to be independent of cell density-dependent killing during acid-challenge (7). We tested an *rpoS* mutant of *E. coli* O157:H7, EK 275 grown under oxidative and fermentative growth conditions. The *rpoS* mutant, grown aerobically was extremely sensitive to acid, and acid sensitivity was independent of cell density during acid-challenge (Figure 2.3a). However, the strain grown fermentatively in LB-glucose showed cell density-dependent survival. Wild type cells survived in a cell density-dependent manner irrespective of aerobic or fermentative growth. Addition of glutamate and arginine rescued the *rpoS* mutant during acid-challenge (Figure 2.3b). Likewise, fermentatively grown wild-type cells (strain EK274) were protected by glutamate in a concentration dependent manner. The acid survival in these experiments was dependent on cell density and the concentration of glutamate and arginine during acid-challenge. This suggests that the glutamate and arginine in the acidified LB medium may have been limiting factors at higher cell densities during acid-challenge. No synergistic protection was observed using a combining arginine and glutamate (data not shown).

b. Role of glutamate- and arginine-dependent acid survival pathways in cell density-dependent acid sensitivity

Next, cell density-dependent acid sensitivity in a *gadC* mutant (strain EK484, defective in glutamate : γ -aminobutyric acid antiporter) was examined. Strain EK484 was grown under two different culture conditions: aerobic growth in LB medium and fermentative growth in LB-glucose medium. The cells were subjected to acid-challenge in the acidified LB medium at various cell densities (Figure 2.4a). The survival of strain EK484, grown aerobically, was independent of cell density, while cells obtained after fermentative growth on LB-glucose broth did exhibit cell density-dependent acid sensitivity. Acid sensitivity of strain EK489, defective in arginine decarboxylase was cell density-dependent irrespective of aerobic or fermentative growth. The arginine decarboxylase pathway would be expected to be induced in strain EK484 when it is grown on LB-glucose. Thus, we determined if arginine could be the limiting factor during acid-challenge for strain EK484 cells that were grown on LB-glucose (Figure 2.4b). Likewise in strain EK489 glutamate decarboxylase pathway is expected to be functional under aerobic as well as fermentative growth conditions.

It was examined if aerobically grown cells of strain EK489 (and wild-type strain EK274) can be rescued during acid-challenge by addition of glutamate. Availability of arginine and glutamate helped cells of strains EK484 and EK489 respectively, to overcome acid-challenge, and there was a clear dose-response relationship between available arginine or glutamate, cell-density and cell survival (Figure 2.4b).

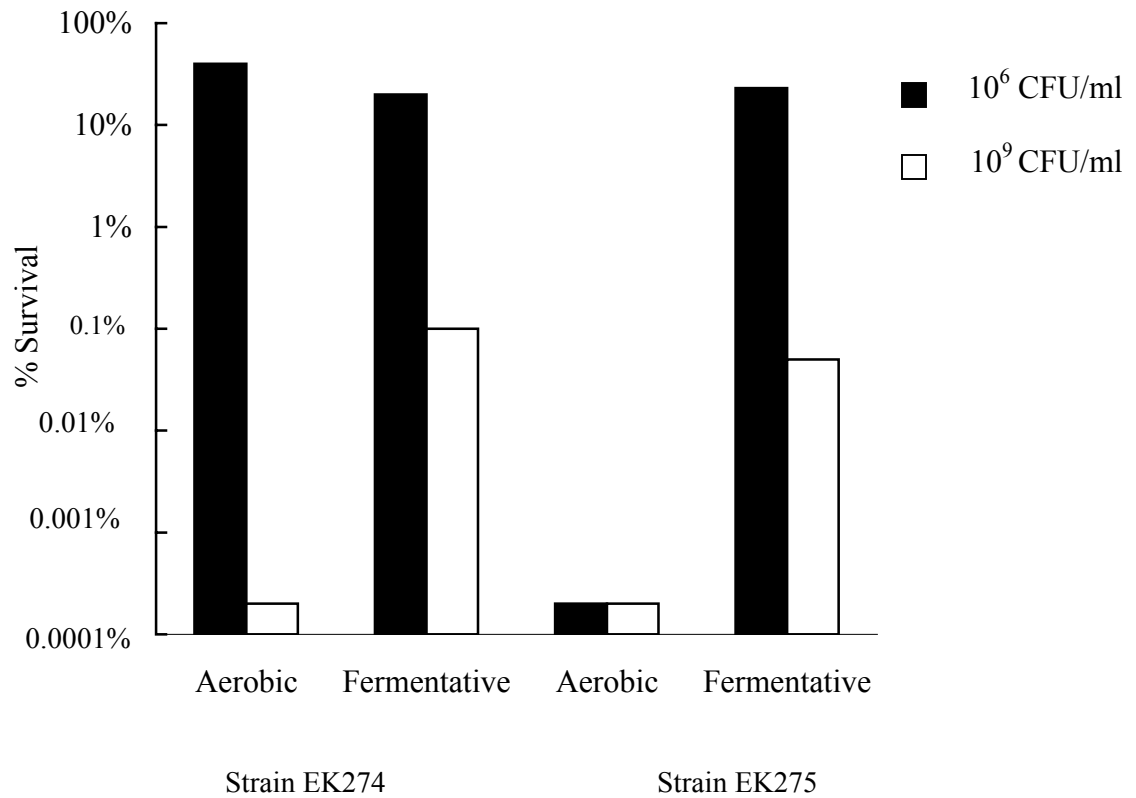


Figure 2.3a. Effect of *rpoS* mutation, and aerobic vs. fermentative growth on survival of *E. coli* O157:H7 at various cell densities in acidified LB (pH 2.5). Aerobic and fermentatively grown cells of *E. coli* O157:H7 strains EK274 and EK275 were subjected to acid-challenge at low (10^6 CFU/ml) and high (10^9 CFU/ml) cell densities. Acid-challenge was performed at pH 2.5, 37 °C for 2 h.

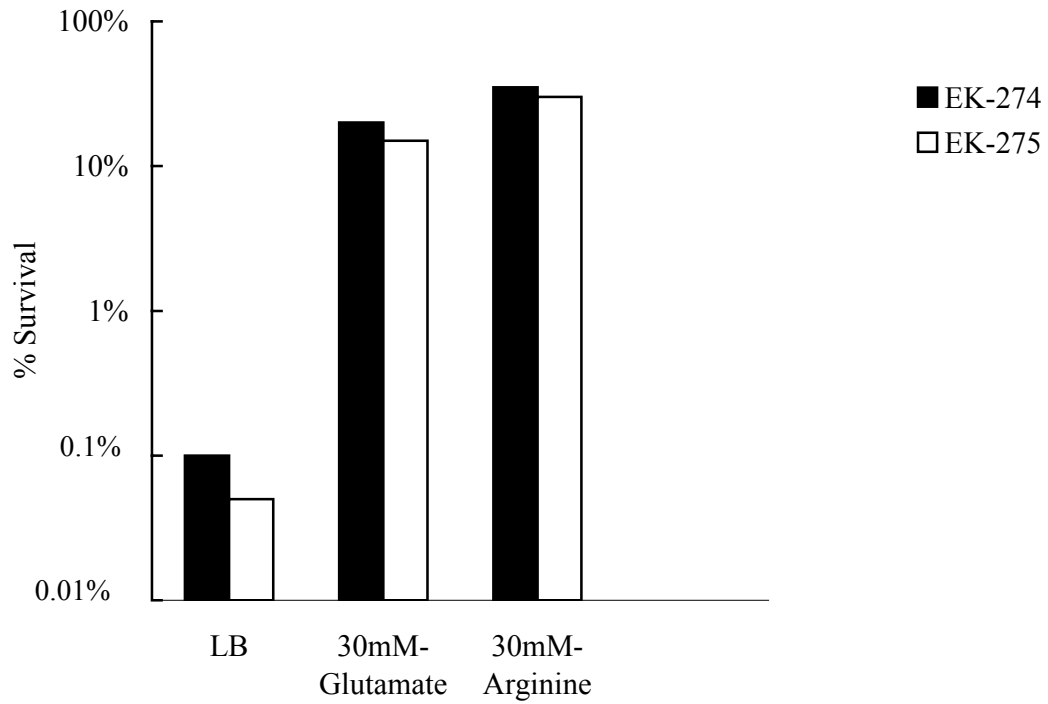


Figure 2.3b. Effect of glutamate and arginine availability during acid-challenge on fermentative growth *rpoS* mutant. Fermentatively grown cells of *E. coli* O157:H7 strains EK275 (open symbols) and EK274 (closed symbols) were subjected to acid-challenge with or without 30 mM glutamate or arginine supplement. Cells were subjected to acid-challenge at high cell density (10^9 CFU/ml).

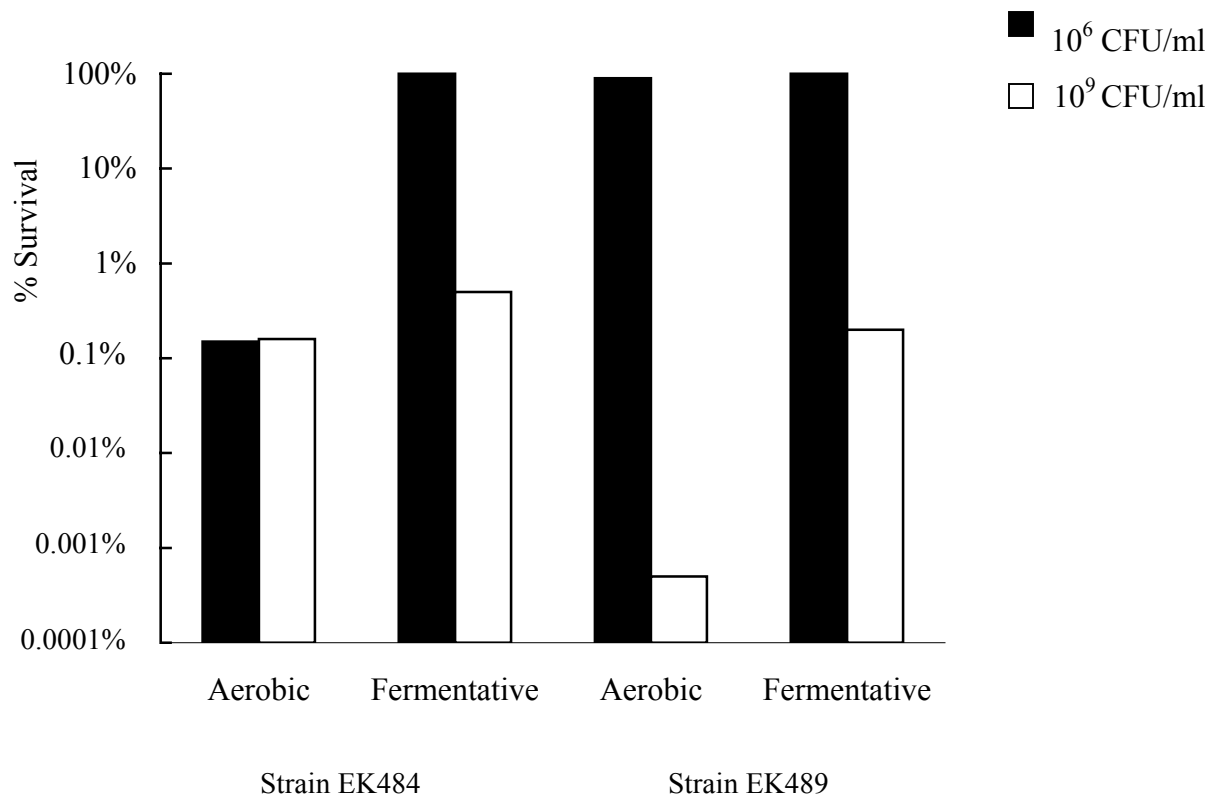


Figure 2.4a. Effect of *gadC* and *adiA* mutations, and aerobic vs. fermentative growth on cell density survival of *E. coli* O157:H7 strains in acidified LB (pH 2.5). EK484 and EK489 were grown aerobically or fermentatively and subjected to acid-challenge at low (10^6 CFU/ml, closed symbols) and high (10^9 CFU/ml, open symbols) cell densities.

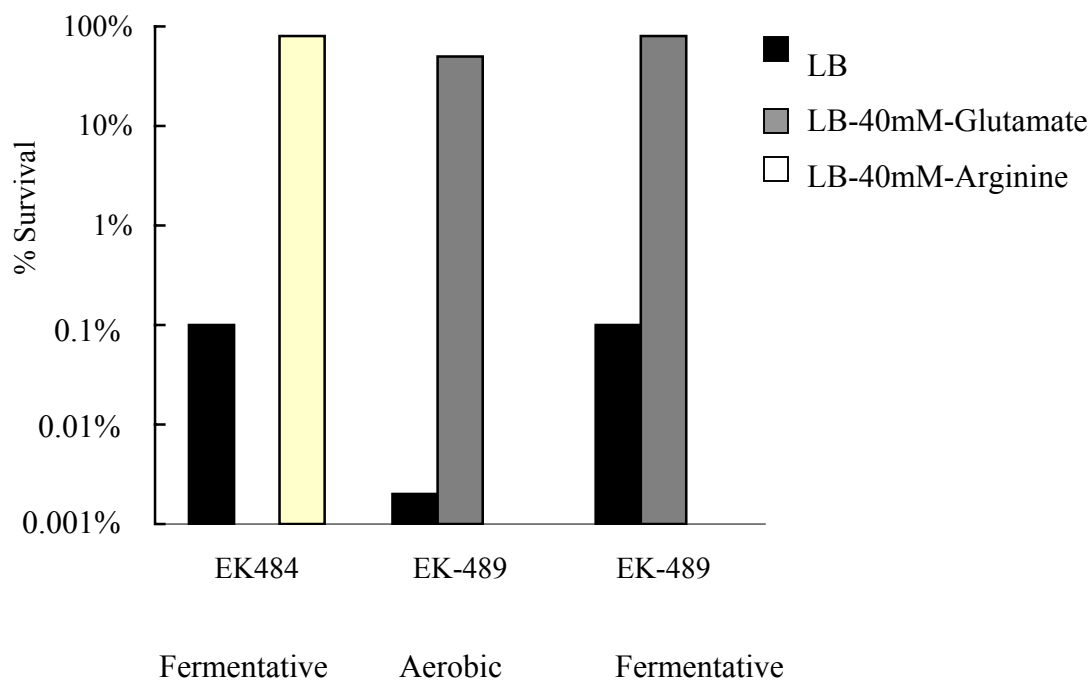


Figure 2.4b. Effect of addition of arginine or glutamate during acid-challenge on survival of *gadC* and *adiA* mutant. EK489 were grown under aerobic and fermentative conditions and EK484 was grown under fermentative condition. Cells were subjected to acid-challenge at high cell density (10^9 CFU/ml) with or without 40 mM glutamate or arginine supplement.

DISCUSSION

Experiments performed to quantitate proposed factors released from high density cell suspensions during acid-challenge indicated that the absence of a factor may be responsible for the cell density-dependent acid sensitivity phenotype (Figure 2.2). Moreover, synthesis of this factor was proposed to be under *rpoS* regulation (7), and indeed, the *rpoS* mutant grown aerobically did not exhibit cell density-dependent acid sensitivity. The likely reason for this is that the *rpoS* mutant, under aerobic growth conditions, does not synthesize glutamate decarboxylase (6), or use glutamic acid-dependent acid survival pathway. As a consequence, the strain has no acid resistant mechanisms, which are operative during aerobic growth. This is reflected in the strain's extreme acid sensitive phenotype.

Activation of the glutamate decarboxylase acid tolerant pathway is complex. It requires functional RpoS as cells enter their stationary growth phase under aerobic conditions, however its activation is RpoS-independent under fermentative growth conditions in the presence of glucose (6). We took advantage of the dual regulatory aspects of glutamate decarboxylase pathway by examining cell density-dependent acid sensitivity of the *rpoS* mutant grown fermentatively. The *rpoS* mutant strain grown on LB-glucose did exhibit the cell density-dependent acid sensitive phenotype (Figure 2.3). This phenotype was most likely due to limited quantities of available glutamate and arginine in the acidified LB medium. Exogenous addition of glutamate (and arginine) during acid-challenge provided protection to cells in a concentration dependent manner.

Limited availability of glutamate (and arginine) causing the cell density-dependent phenotype was further confirmed by using *gadC* and *adiA* mutant strains. In addition to the defect in the glutamate decarboxylase pathway, aerobically grown *gadC* mutant cells do not utilize arginine decarboxylase pathway (17). Thus, the aerobically grown *gadC* mutant has only an *rpoS*-mediated oxidative acid survival pathway that is functional when challenged in the acidified LB medium. Since the strain did not exhibit cell density-dependent acid sensitivity, the *rpoS*-mediated acid survival pathway appears to be independent of glutamate and arginine availability during acid-challenge. On the other hand, cells grown under fermentative growth possessed a fully activated arginine decarboxylase pathway, and did exhibit cell density-dependent acid sensitivity. This was confirmed by the addition of increasing amounts of arginine, which helped the cells survive even under high cell density populations in a concentration dependent manner. This indicated that arginine is probably one of the limiting components in the acidified LB medium responsible for cell density-dependent acid sensitivity.

S. Typhimurium cells do not possess glutamate or arginine decarboxylase mediated acid resistant mechanisms (3), and we confirmed the absence of glutamate- or arginine-dependent acid sensitivity in this organism (data not shown). Contrary to the previous report (7), the wild type *E. coli* strain K-12 (MG1665) which possessed all three acid resistant pathways, like several other O157:H7 strains, showed cell density-dependent acid sensitivity.

The impact of low pH on the virulence of *Salmonella* and *E. coli* strains is profound. Studies suggest that acid tolerance aids in survival and pathogenesis of organisms since several mutants defective in acid tolerance were attenuated in virulence.

A possible link between low pH and cell-to-cell communication has been described in that *Salmonella* and *E. coli* strains grown at a low pH synthesize an autoinducer similar to the system 2 autoinducer involved with induction of bioluminescence in *Vibrio harveyi* (26, 27). Moreover, induction of the type III secretion system in enterohemorrhagic *E. coli* O157:H7 has been demonstrated recently to be under the control of quorum sensing molecules (25). Thus, there may exist yet to be proven links between quorum sensing and acid tolerance in enteric bacteria (8). Characterization of such a link will significantly advance in our understanding of cell-to-cell communication in low pH environments and its influence on virulence.

REFERENCES

1. **Abdul-Raouf, U. M., L. R. Beuchat, and M. S. Ammar.** 1993. Survival and growth of *Escherichia coli* O157:H7 in ground, roasted beef as affected by pH, acidulants, and temperature. *Appl Environ Microbiol.* **59**:2364-8.
2. **Arnold, K. W., and C. W. Kaspar.** 1995. Starvation- and stationary-phase-induced acid tolerance in *Escherichia coli* O157:H7. *Appl Environ Microbiol.* **61**:2037-9.
3. **Bang, I. S., B. H. Kim, J. W. Foster, and Y. K. Park.** 2000. OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar typhimurium. *J Bacteriol.* **182**:2245-52.
4. **Bearson, S., B. Bearson, and J. W. Foster.** 1997. Acid stress responses in enterobacteria. *FEMS Microbiol Lett.* **147**:173-80.
5. **Beumer, R. R., J. de Vries, and F. M. Rombouts.** 1992. *Campylobacter jejuni* non-culturable coccoid cells. *Int J Food Microbiol.* **15**:153-63.
6. **Castanie-Cornet, M. P., T. A. Penfound, D. Smith, J. F. Elliott, and J. W. Foster.** 1999. Control of acid resistance in *Escherichia coli*. *J Bacteriol.* **181**:3525-35.

7. **Datta, A. R., and M. M. Benjamin.** 1999. Cell density dependent acid sensitivity in stationary phase cultures of enterohemorrhagic *Escherichia coli* O157:H7. FEMS Microbiol Lett. **181**:289-95.
8. **De Kievit, T. R., and B. H. Iglewski.** 2000. Bacterial quorum sensing in pathogenic relationships. Infect Immun. **68**:4839-49.
9. **Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J. Harwood, and D. G. Guiney.** 1992. The alternative sigma factor katF (rpoS) regulates *Salmonella* virulence. Proc Natl Acad Sci U S A. **89**:11978-82.
10. **Foster, J. W.** 1999. When protons attack: microbial strategies of acid adaptation. Curr Opin Microbiol. **2**:170-4.
11. **Foster, J. W., and H. K. Hall.** 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella* Typhimurium. J Bacteriol. **173**:5129-35.
12. **Foster, J. W., and M. Moreno.** 1999. Inducible acid tolerance mechanisms in enteric bacteria. Novartis Found Symp. **221**:55-69.
13. **Glass, K. A., J. M. Loeffelholz, J. P. Ford, and M. P. Doyle.** 1992. Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. Appl Environ Microbiol. **58**:2513-6.
14. **Gorden, J., and P. L. Small.** 1993. Acid resistance in enteric bacteria. Infect Immun. **61**:364-7.
15. **Hersh, B. M., F. T. Farooq, D. N. Barstad, D. L. Blankenhorn, and J. L. Slonczewski.** 1996. A glutamate-dependent acid resistance gene in *Escherichia coli*. J Bacteriol. **178**:3978-81.
16. **Hussain, N. H., M. Goodson, and R. J. Rowbury.** 1998. Recent advances in biology: intercellular communication and quorum sensing in micro-organisms. Sci Prog. **81**:69-80.
17. **Lin, J., I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster.** 1995. Comparative analysis of extreme acid survival in *Salmonella* Typhimurium, *Shigella flexneri*, and *Escherichia coli*. J Bacteriol. **177**:4097-104.
18. **O'Hara, G. W., and A. R. Glenn.** 1994. The adaptive acid tolerance response in root nodule bacteria and *Escherichia coli*. Arch Microbiol. **161**:286-92.
19. **Price, S. B., C. M. Cheng, C. W. Kaspar, J. C. Wright, F. J. DeGraves, T. A. Penfound, M. P. Castanie-Cornet, and J. W. Foster.** 2000. Role of rpoS in acid resistance and fecal shedding of *Escherichia coli* O157:H7. Appl Environ Microbiol. **66**:632-7.

20. **Raja, N., M. Goodson, W. C. Chui, D. G. Smith, and R. J. Rowbury.** 1991. Habituation to acid in *Escherichia coli*: conditions for habituation and its effects on plasmid transfer. *J Appl Bacteriol.* **70**:59-65.
21. **Rao, N. N., S. Liu, and A. Kornberg.** 1998. Inorganic Polyphosphate in *Escherichia coli*: the Phosphate Regulon and the Stringent Response. *J. Bacteriol.* **180**:2186-2193.
22. **Rowbury, R. J.** 1999. Acid tolerance induced by metabolites and secreted proteins, and how tolerance can be counteracted. *Novartis Found Symp.* **221**:93-106.
23. **Rowbury, R. J.** 2000. Killed cultures of *Escherichia coli* can protect living organisms from acid stress. *Microbiol.* **146**:1759-60.
24. **Small, P., D. Blankenhorn, D. Welty, E. Zinser, and J. L. Slonczewski.** 1994. Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of *rpoS* and growth pH. *J. Bacteriol.* **176**:1729-1737.
25. **Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper.** 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proc Natl Acad Sci U S A.* **96**:15196-201.
26. **Surette, M. G., and B. L. Bassler.** 1999. Regulation of autoinducer production in *Salmonella* Typhimurium. *Mol Microbiol.* **31**:585-95.
27. **Surette, M. G., M. B. Miller, and B. L. Bassler.** 1999. Quorum sensing in *Escherichia coli*, *Salmonella* Typhimurium, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. *Proc Natl Acad Sci U S A.* **96**:1639-44.

Chapter 3 Rapid Sample Preparation Method for PCR-based Detection of *Escherichia coli* O157:H7 in Ground Beef

ABSTRACT

Infection with *E. coli* O157:H7 and other Shiga toxin-producing *E. coli* (STEC) can result in hemorrhagic colitis, hemolytic uremic syndrome, and death. *E. coli* O157:H7 causes 73,480 illnesses, 2,168 hospitalizations, and 61 deaths each year in the United States, approx. 85% of the cases result from the ingestion of contaminated food. Contaminated ground beef has been identified as the main source of infection in *E. coli* O157:H7 outbreaks. To develop an improved, rapid and sensitive sample preparation method for PCR-based detection of *Escherichia coli* O157:H7 in ground beef, fresh ground beef samples were experimentally inoculated with varying concentrations of *E. coli* O157:H7. PCR inhibitors were removed and bacterial cells were concentrated by filtration and centrifugation, and lysed using enzymatic digestion and successive freeze/thaw cycles. DNA was purified and concentrated via phenol/chloroform extraction and the Shiga toxin 1 gene (*stx1*) was amplified using PCR to evaluate the sample preparation method. Without prior enrichment of cells in broth media, the detection limit was 10^3 CFU g⁻¹ beef. When a 6-h enrichment step was incorporated, the detection limit was 10^0 CFU g⁻¹ beef. Total time required from beginning to end of the procedure is 12 h. The sample preparation method developed here enabled substantially improved sensitivity in the PCR-based detection of *E. coli* O157:H7 in ground beef, as compared to previous reports. Superb sensitivity, coupled with quick turn-around time, relative ease of

use, and cost-effectiveness, makes this a useful method for detecting *E. coli* O157:H7 in ground beef.

INTRODUCTION

In humans, infection with *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* (STEC) can result in hemorrhagic colitis, hemolytic uremic syndrome, and death (11). The principal route of infection is via the food chain. Among the estimated 73,480 illnesses, 2,168 hospitalizations, and 61 deaths caused each year in the United States by *E. coli* O157:H7, approx. 85% are thought to occur through ingestion of contaminated food (13). *E. coli* O157:H7 was first recognized as a pathogen following a 1982 outbreak of food-borne illness associated with the consumption of undercooked ground beef (17). Since that time, cattle have been implicated as a principal reservoir of *E. coli* O157:H7 (14). For instance, in a study analyzing feces, hides, and carcasses of beef cattle during processing, as much as 28% of cattle fecal samples were positive for *E. coli* O157:H7 (8). Accordingly, contaminated ground beef has been identified as the source of infection in 48 of 196 *E. coli* O157:H7 outbreaks documented in the United States between 1982 and 1998 (3,14). Most recently, the United States government ordered the second largest recall of beef in the U.S. history (approx. 19 million pounds of beef were recalled from 21 states) after at least 16 people became ill from eating beef contaminated with *E. coli* O157:H7 (4).

Conventional enrichment and isolation methods with selective and/or indicator media, such as *E. coli* (EC) broth, lauryl sulfate tryptose 4-methylumbellifery- β -D-glucuronic acid broth, eosin methylene blue agar, and MacConkey sorbitol agar, are

currently used to detect *E. coli* O157:H7 in ground beef. However, such tests often require several days to get results, during which time meat may be shipped from production plants to retail stores. These tests also lack the ability to identify *E. coli* O157:H7 strains definitively (1). Thus development of detection methods requiring less time is important in preventing distribution of contaminated beef from producer to consumer. Because *E. coli* O157:H7 strains that cause serious disease produce at least one Shiga toxin (Stx1 and/or Stx2) (15), detection of Shiga toxin genes by PCR is very useful for detecting *E. coli* O157:H7.

But the contamination level of *E. coli* O157:H7 in ground beef samples is usually quite low, i.e., less than 100 CFU g⁻¹ beef (14). In addition, a variety of substances in beef samples, blood and food particles for instance, are potent inhibitors of the PCR (3, 6). As a result, small variation in amount of inhibitors can lead to large variation in PCR amplification. Techniques such as centrifugation, filtration, DNA affinity columns, and magnetic beads coated with specific antibodies or lectins (12, 18, 20), which concentrate pathogens or total nucleic acids, and remove PCR inhibitors, have shown promise when applied to ground beef samples. Moreover, improvement in efficiency of procedures for lysing cells and harvesting target DNA may further increase sensitivity of detection assays (1,2).

We report development of a rapid, sensitive PCR template preparation method to remove amplification inhibitors and concentrate *E. coli* O157:H7 cells from ground beef samples. Based on subsequent PCR amplification of the *stx1* gene, 10⁰ CFU *E. coli* O157:H7 g⁻¹ ground beef could be reproducibly detected in 12 h. Superb sensitivity,

quick turn-around time, relative ease of use, and cost-effectiveness make this a valuable method for detecting *E. coli* O157:H7 in ground beef.

MATERIALS AND METHODS

Bacterial strains and growth conditions

E. coli O157:H7 strain ATCC 932 NA^r (resistant to 50 µg ml⁻¹ nalidixic acid) was used in all experiments because the NA^r phenotype enabled easy enumeration of *E. coli* O157:H7 cells in ground beef samples. The strain was grown in 5 ml of Luria-Bertani (LB) broth medium (Sigma, St. Louis, MO, USA), containing 50 µg ml⁻¹ nalidixic acid (Sigma), at 37°C in 50 ml flasks with rotary agitation (230 rpm min⁻¹) for 16 to 18 h. Stationary phase cells were harvested by centrifugation at 6,500 × *g* for 6 min at room temperature. Cell pellets were washed in 15 ml of saline and re-suspended in saline to an O.D.₆₀₀ of 0.5 (approx. 1 × 10⁹ CFU ml⁻¹). Different cell concentration solutions, as determined via plate counts on LB Agar (Sigma), were used in subsequent experiments.

Sample Preparation Method

Packages of fresh ground beef were purchased from retail supermarkets. Ground beef samples (25 grams each) were spiked with different concentrations of *E. coli* O157:H7 and homogenized in 225 ml of saline at 230 rev min⁻¹ for 90 s in a two-chamber filter bag (Fisher Scientific, Pittsburgh, PA, USA) using a Lab-Blender 400 stomacher (Seward Laboratory, London, UK). Homogenized samples were centrifuged at 50 × *g*, 100 × *g*, 200 × *g*, or 400 × *g* for 10 min. Resultant supernatants were placed in fresh tubes and centrifuged at 6,500 × *g* for 15 min, after which the pellets were re-suspended

in 25 ml of saline. At this point, to evaluate the cell recovery rate, cell concentrations were determined by plate counts using MacConkey sorbitol agar containing 50 $\mu\text{g ml}^{-1}$ nalidixic acid. Samples were centrifuged again at $6,500 \times g$ for 15 min and resultant pellets were used to extract DNA.

PCR Template Preparation

a. The boiling method

Samples spiked with different concentrations of *E. coli* O157:H7 cells in saline, or in ground beef-saline (1:10, w/v) solutions, were boiled at 100 °C for 10 min. Insoluble material was removed by centrifugation for 2 min and the supernatant used as template in the PCR.

b. The enzyme and freeze/thaw method

Nucleic acids were extracted from pellets by a direct lysis procedure adapted from previously described methods (2,4,5, 21). The pellets were re-suspended in 2 ml of buffer A (500 mmol l^{-1} Tris-HCl [pH 8.0], 100 mmol l^{-1} NaCl, 1 mmol l^{-1} sodium citrate, 5 mmol l^{-1} EDTA) in the presence of 5 mg ml^{-1} lysozyme, and incubated for 1 h at 37°C with occasional agitation. Proteinase K was added to a final concentration of 2 mg ml^{-1} , and the mixture incubated for an additional 30 min with occasional agitation. Following incubation, 1.33 ml of lysis buffer (500 mmol l^{-1} Tris-HCl [pH 8.0], 100 mmol l^{-1} NaCl, 4% [w/v] SDS, 25 mmol l^{-1} EDTA) were added and the solution mixed gently by inversion. Three cycles of freezing in a dry ice-ethanol bath and thawing in a 65°C water bath were included to facilitate cell wall destruction, and thus, release of nucleic acids.

The mixture (1ml) was used to extract DNA with an equal volume of phenol (pH 6.7 ± 0.2, Sigma), followed by extraction with an equal volume of chloroform. Nucleic acids were precipitated via addition of an equal volume of isopropyl alcohol, and centrifuged at maximum speed in a microcentrifuge for 20 min. Pellets were washed with 70% ethanol and air-dried at 37°C for 5 min, after which they were dissolved in 50 µl distilled water and stored at -30°C until further use.

c. PCR Amplification of the *stxI* gene

The *stxI* gene was amplified by PCR using a slight modification of a previously published method (16). Template DNA (5 µl) was added to 45µl of a reaction mixture containing 0.1 mmol l⁻¹ each deoxyribonucleotide, 10 mmol l⁻¹ Tris-HCl (pH 8.3), 50 mmol l⁻¹ KCl, 10 mmol l⁻¹ Na₂EDTA, 3 mmol l⁻¹ MgCl₂, and 1.0 U *AmpliTaq* Gold DNA polymerase (Applied Biosystems, Branchburg, NJ, USA). The oligonucleotide primers used to amplify the *stxI* gene segment were F5'-ACGTTACAGCGTGTGCTGGGATC-3' and R5'-TTGCCACAGACTGCGTCAGTTAGG-3'. The primers were predicted to amplify a 123-bp fragment of the *stxI* gene. The PCR was done using a GeneAmp PCR system 9700 thermal cycler (Perkin-Elmer, Foster City, CA, USA). The program consisted of an initial template denaturation step at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1min, and primer extension at 72°C for 1 min. A final extension step was performed at 72°C for 7 min. Amplified products were electrophoresed in 2% agarose gels containing 0.5 µg ml⁻¹ ethidium bromide. Appropriate controls were included, such as buffer alone and unspiked ground beef samples were included as negative controls, whereas saline or

ground beef samples spiked with high concentration of *E. coli* O157:H7 were used as positive controls.

Enrichment of *E. coli* O157:H7 from ground beef

Ground beef samples (25 grams each) were spiked with 10^0 CFU *E. coli* O157:H7 g⁻¹ beef. Samples were homogenized in 225 ml pre-warmed modified EC medium base (DIFCO, Becton Dickinson, Sparks, MD, USA) at 230 rev min⁻¹ for 90 s in stomach bags (Seward) without a filter. Samples were incubated at 37°C in 500 ml flasks with rotary agitation (230 rpm min⁻¹) for 4 or 6 h, after which they were further homogenized at 230 rpm min⁻¹ for 30 s in two-chamber filter bags. Template preparation was then done as by using enzyme and freeze/thaw method.

RESULTS

The effect of ground beef material on the PCR

To assess the effect of ground beef substances on the PCR, different concentrations of *E. coli* O157:H7 cells were suspended in pure saline or a mixture of ground beef and saline (1:10, w/v). PCR templates were then prepared by boiling method as described above. The detection limit for cells suspended in pure saline was 1.34×10^5 CFU ml⁻¹ (Figure 3.1, lane 5), whereas the detection limit for cells suspended in the ground beef/saline mixture was 3.4×10^{10} CFU ml⁻¹ (Figure 3.1, lane 11). Thus there was approximately 2.5×10^5 times difference in the number of cells required for PCR detection of the *stx1* gene in saline as compared to the cells suspended in the ground beef/saline mixture.

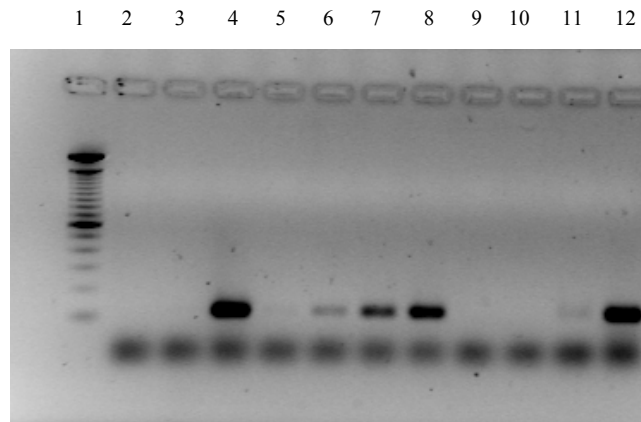


Figure 3.1. PCR amplification of *stx1* gene for detecting *E. coli* O157:H7 using templates prepared by boiling method from saline and saline/ground beef (1:10, wt/wt) solutions spiked with different levels of *E. coli* O157:H7. Lane 1, 123-bp DNA marker; lane 2, PCR buffer control; lane 3, saline control; lane 4, *E. coli* O157:H7 positive control; lane 5, 1.48×10^5 CFU ml⁻¹ in saline; lane 6, 1.48×10^6 CFU ml⁻¹ in saline; lane 7, 1.48×10^7 CFU ml⁻¹ in saline; lane 8, 1.48×10^8 CFU ml⁻¹ in saline; lane 9, saline/ground beef solution control; lane 10, 3.4×10^8 CFU g⁻¹ in saline/ground beef solution; lane 11, 3.4×10^9 CFU g⁻¹ in saline/ground beef solution; lane 12, 3.4×10^{10} CFU g⁻¹ in saline/ground beef solution.

The effect of centrifugation on *E. coli* O157:H7 cell recovery

After homogenization and filtration, ground beef samples were processed by centrifugation as described above. The recovery rate of *E. coli* O157:H7 using different first step centrifugation forces was tested. There was no difference between the recovery rate when centrifuging $50 \times g$ or $100 \times g$ centrifugation forces; however, both resulted in higher cell recovery than when centrifuging at $200 \times g$ and $400 \times g$ (data not shown). In addition, when using a $100 \times g$ centrifugation force, more food particles were removed than when using a $50 \times g$ force. Hence a $100 \times g$ first-step centrifugation was included in the protocol. Thereafter, centrifugation at $6,500 \times g$ resulted in a 0.5 to 1.0 g pellet from 25 g of ground beef. The concentration of *E. coli* O157:H7 cells in the pellet, as determined via plate counts on MacConkey sorbitol agar containing $50 \mu\text{g ml}^{-1}$ nalidixic acid, indicated 75% ($N = 9$, S.D. = 9%) of the cells were recovered. Even for very low inoculation level, e.g., 10^3 CFU *E. coli* O157:H7 g^{-1} beef, 75% of cells were recovered in the pellet, perhaps because small food particles helped to pellet the bacteria. Regardless, concentrations of cells in the pellets were increased 18.8 to 37.5 times over those in ground beef.

The effect of sample preparation method on PCR detection of *E. coli* O157:H7

Using enzyme digestion followed by freeze/thaw cycles and phenol-chloroform extraction to prepare PCR templates, as few as 10 to 100 CFU *E. coli* O157:H7 cells g^{-1} beef could be detected (Figure 3.2a); these results were not consistently repeatable. Use of this method did enable consistently repeatable detection of 1000 CFU *E. coli* O157:H7 cells g^{-1} beef (Figure 3.2b).

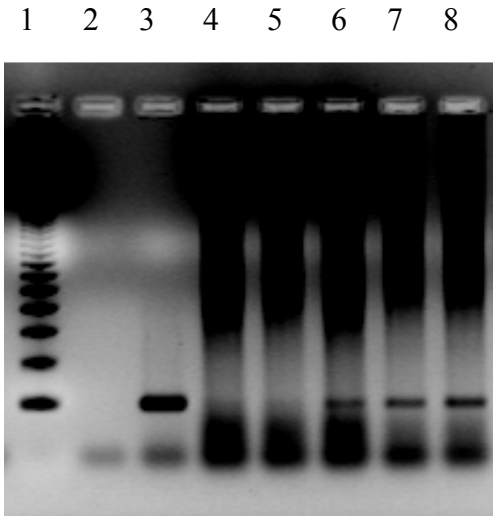


Figure 3.2a. PCR amplification of *stx1* gene for detecting *E. coli* O157:H7 using templates prepared from beef samples spiked with different levels of *E. coli* O157:H7. Lane 1, 123-bp DNA marker; lane 2, PCR buffer control; lane 3, *E. coli* positive control; lane 4, ground beef control; lane 5, 10^0 CFU g^{-1} ; lane 6, 10^1 CFU g^{-1} ; lane 7, 10^2 CFU g^{-1} ; lane 8, 10^4 CFU g^{-1} .

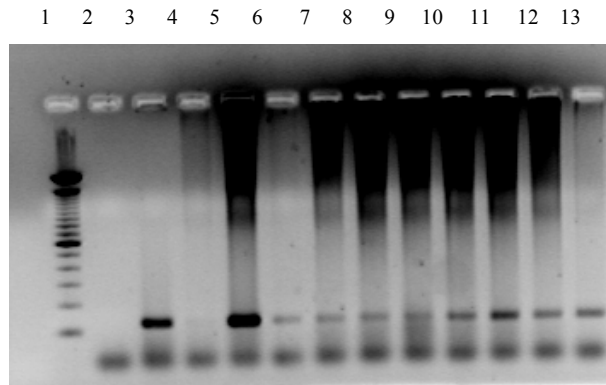


Figure 3.2b. PCR amplification of *stx1* gene for detecting *E. coli* O157:H7 using templates prepared from beef samples spiked with different levels of *E. coli* O157:H7. Lane 1, 100-bp DNA marker; lane 2, PCR buffer control; lane 3, *E. coli* positive control; lane 4, beef control; lane 5, *E. coli* positive ground beef control; lanes 6 through 9, 10^3 CFU g^{-1} ; lanes 10 through 13, 10^4 CFU g^{-1} .

The effect of enrichment on PCR detection of *E. coli* O157:H7

To improve the sensitivity of detection, an enrichment step was added. Ground beef samples spiked with 10^0 CFU *E. coli* O157:H7 g^{-1} of beef were enriched in modified EC medium base for 4 or 6 h. PCR templates were prepared by using enzyme and freeze/thaw method as described above. After 4 h of enrichment, the *stx1* gene could be detected by PCR, but the results were not consistently repeatable. After a 6-h enrichment step, however, the *stx1* gene was consistently and reproducibly detected in all samples, but not in the ground beef *E. coli* O157:H7-free control samples (Figure. 3.3). As determined by viable counts, the concentration of *E. coli* O157:H7 cells was 10^2 CFU g^{-1} beef after 4 h enrichment, whereas the concentration of *E. coli* O157:H7 cells was 10^4 CFU g^{-1} beef after 6 h enrichment (data not shown).

DISCUSSION

Using the protocol developed in this study, 10^3 CFU *E. coli* O157:H7 g^{-1} beef were concentrated and reproducibly detected by PCR, without an enrichment step. From beginning to end, the time required to conduct this procedure was roughly six hours. To the best of our knowledge, this represents the most sensitive detection limit for a PCR-based method without enrichment. An enrichment step was incorporated to heighten the sensitivity of the assay. Enrichment and PCR methods typically require a 12- to 24-h enrichment in order to detect 10^0 CFU *E. coli* O157:H7 g^{-1} ground beef (22).

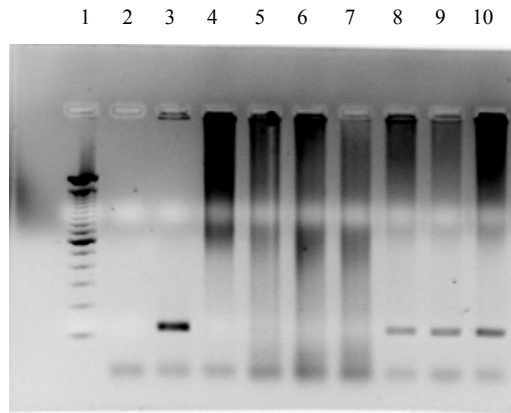


Figure 3.3. PCR amplification of *stx1* gene for detecting *E. coli* O157:H7 using templates prepared from beef samples after 4 or 6 h enrichment spiked with 10^0 CFU g^{-1} of *E. coli* O157:H7. Lane 1, 100-bp DNA marker; lane 2, PCR buffer control; lane 3, *E. coli* positive control; lane 4, ground beef control after 6 h enrichment; lanes 5 through 7, 10^0 CFU g^{-1} after 4 h enrichment; lanes 8 through 10, 10^0 CFU g^{-1} after 6 h enrichment

Using the sample preparation method described here, we were able to detect 10^0 CFU *E. coli* O157:H7 g^{-1} ground beef after enrichment for 6 hours. Thus the total time required for reliably detecting 10^0 CFU *E. coli* O157:H7 g^{-1} ground beef was 12 h, a substantial decrease in time as compared to previous reports. The relatively short enrichment step was advantageous in that it lessened the time for normal microbiota to out-compete indicator bacteria (7), a problem that is particularly problematic regarding *E. coli* O157:H7 and other pathogens for which the infectious dose is low (9). Because the ground beef samples were thoroughly homogenized, use of this protocol reduced the chance that *E. coli* cells remained undetected due to protection within food matrices.

Similar to the report by Witham (22), we observed a difference in the sensitivity of the PCR based on sample preparation methods. Specifically, the detection limit of the PCR was much greater for bacteria suspended in saline, as compared to those suspended in a mixture of ground beef and saline. The efficiency of cell lysis and the procedures for harvesting target DNA are also important factors that can limit the sensitivity of the PCR assay. For example, the extra time invested in a phenol/chloroform extraction (approx. 1 h) resulted in a substantial dividend: as few as 1.6×10^2 CFU *E. coli* O157:H7 cells per reaction could be detected via PCR, whereas when sample preparation consisted of only boiling in saline, the detection limit was 1.6×10^4 CFU per reaction (22).

Previous research has shown that lysozyme and proteinase K are powerful enzymes for lysing bacteria (2, 5). But the presence of either lysozyme or proteinase K in the PCR reactions, even after their denaturation at 99.5°C , may substantially hinder DNA amplification. The presence of detergents or other lysis reagents may also affect the efficiency of DNA amplification. Maximum amplification, therefore, is dependent upon

purification of target DNA (2). Among DNA purification methods, ether extraction is known to be effective for removing PCR inhibitors present in the samples (19). In this study, a variety of physical and chemical agents, including lysozyme, proteinase K, detergents, freezing and thawing, and phenol-chloroform extraction, were used to facilitate cell lysis and concentrate DNA.

The sample preparation method described in this study was relatively easy to perform and the total cost of analysis (not including thermal cycler) was roughly equal that of conventional enrichment and culture methods. Besides this, 2-3 hours could be further reduced by using real-time PCR or other DNA amplification technologies such as strand displacement amplification (10) and ramification amplification. However, this method is not specific for a particular organism and thus may be useful for detecting other bacterial pathogens in ground beef; it should also be useful for other DNA amplification-dependent assays, e.g., SDA or biosensors. Whether or not this method is applicable for other types of foods remains to be determined. Nevertheless, the results described above indicate this method can be used to achieve high sensitivity for detection of *E. coli* O157:H7 in ground beef in a relatively short period of time. The continued advent of novel techniques such as real-time PCR foreshadows further reduction in the time required for this and similar methods for detection of food-borne bacterial pathogens.

REFERENCES

1. USDA/FSIS Microbiology Laboratory Guidebook, 1998. 3rd Edition, 5.1-5.10.
2. **Abolmaaty, A., El-Shemy, M.G., Khallaf, M.F. and Levin, R.E.** 1998. Effect of lysing methods and their variables on the yield of *Escherichia coli* O157:H7 DNA and its PCR amplification. J. Microbiol. Methods 34:133-141.
3. **Abu Al-Soud, W., and P. Radstrom.** 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. J Clin Microbiol 38:4463-70.
4. United States Department of Agriculture. Recall Notification Report. <http://www.fsis.usda.gov/OA/recalls/rnrfiles/rnr055-2002.htm> 055-2002. 2002. Food Safety and Inspection Service, United States Department of Agriculture, Washington, DC. Accessed April 12, 2004.
5. **Barns, S. M., R. E. Fundyga, M. W. Jeffries, and N. R. Pace.** 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. Proc Natl Acad Sci U S A 91:1609-13.
6. **Bhaduri, S. a. C., B.** 2001. Sample preparation methods for PCR detection of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on beef chuck shoulder using a single enrichment medium. Mol. Cell Probes 15:267-274.
7. **Clark, J. A.** 1980. The influence of increasing numbers of non-indicator organisms upon the detection of indicator organisms by the membrane filter and presence-absence tests. Can. J. Microbiol. 26:827-832.
8. **Elder, R. O., J. E. Keen, G. R. Siragusa, G. A. Barkocy-Gallagher, M. Koohmaraie, and W. W. Laegreid.** 2000. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. Proc Natl Acad Sci U S A 97:2999-3003.
9. **Feng, P.** 2001. Development and impact of rapid methods for detection of foodborne pathogens. In Food microbiology: fundamentals and frontiers. ASM Press, Washington, DC.
10. **Ge, B., C. Larkin, S. Ahn, M. Jolley, M. Nasir, J. Meng, and R. H. Hall.** 2002. Identification of *Escherichia coli* O157:H7 and other enterohemorrhagic serotypes by EHEC- hly A targeting, strand displacement amplification, and fluorescence polarization. Mol Cell Probes 16:85-92.

11. **Griffin, P. M.** 1995. *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli*. In Infections of the gastrointestinal tract. Raven Press, Ltd, New York.
12. **Kaclikova, E., Kuchta, T.V., Kay, H. and Gray, D.** 2001. Separation of *Listeria* from cheese and enrichment media using antibody-coated microbeads and centrifugation. *J. Microbiol. Methods* 46:63-67.
13. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. *Emerg Infect Dis* 5:607-25.
14. **Meng, J., Doyle, M. P., Zhao, T. and Zhao, S.** 2001. Enterohemorrhagic *Escherichia coli*. In Food microbiology: fundamentals and frontiers. ASM Press, Washington, DC.
15. **O'Brien, A. D., V. L. Tesh, A. Donohue-Rolfe, M. P. Jackson, S. Olsnes, K. Sandvig, A. A. Lindberg, and G. T. Keusch.** 1992. Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. *Curr Top Microbiol Immunol* 180:65-94.
16. **Pass, M. A., R. Odedra, and R. M. Batt.** 2000. Multiplex PCRs for identification of *Escherichia coli* virulence genes. *J Clin Microbiol* 38:2001-4.
17. **Riley, L. W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A. and Cohen, M.L.** 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N. Engl. J. Med* 308:681-685.
18. **Seo, K. H., R. E. Brackett, J. F. Frank, and S. Hilliard.** 1998. Immunomagnetic separation and flow cytometry for rapid detection of *Escherichia coli* O157:H7. *J Food Prot* 61:812-6.
19. **Simon M.C., G. D. I., and Cook N.** 1996. DNA extraction and PCR methods for the detection of *Listeria monocytogenes* in cold-smoked salmon. *Appl. Environ. Microbiol.* 62:822-824.
20. **Tomoyasu, T.** 1998. Improvement of the immunomagnetic separation method selective for *Escherichia coli* O157 strains. *Appl. Environ. Microbiol* 64:376-382.
21. **Tsai, Y. L. a. O., B.H.** 1991. Rapid method for direct extraction of DNA from soil and sediments. *Appl. Environ. Microbiol* 57:1070-1074.
22. **Witham, P. K., Yamashiro, C.T., Livak, K.J. and Batt, C.A.** 1996. A PCR-based assay for the detection of *Escherichia coli* Shiga-like toxin genes in ground beef. *Appl. Environ. Microbiol.* 62:1347-1353.

Chapter 4 Development of a Rapid Method for Isolating *Salmonella* from Poultry Products

ABSTRACT

Poultry products are one of the major sources of *Salmonella* infections in human beings. Although many methods for isolating *Salmonella* from food and clinical samples are available, none has been designed specifically based on unique characteristics of poultry products. An improved procedure based on the method described in the USDA/Food Safety Inspection Service (FSIS) Microbiological Laboratory Guidebook was developed to isolate *Salmonella* from poultry products. The modified method was evaluated using the standard procedures of the FDA/Bacteriological Analytical Manual (BAM) and USDA/FSIS Microbiological Laboratory Guidebook. Greater than 95% of the presumptive *Salmonella* colonies isolated using the modified method from chicken samples were confirmed as *Salmonella* by PCR/ API 20E tests. Its high specificity significantly reduced the time for isolation and identification of *Salmonella* from poultry products (48 h compared to 3-5 days); thus, the costs were reduced. Since the method has a high specificity, *Salmonella* isolates may be further subtyped by serotyping and/or pulsed-field gel electrophoresis (PFGE) without biochemical confirmation. Using this method, we were able to recover *Salmonella* from 29 of 44 (66%) retail organic chicken samples. The widespread occurrence of *Salmonella* in organic chicken samples emphasizes the need for implementation of effective control measures of microbial contaminations in organic poultry products.

INTRODUCTION

Salmonella is one of the leading causes of foodborne infections worldwide (11, 23, 24, 28). Poultry products are widely acknowledged to be a significant reservoir for *Salmonella* and have frequently been incriminated as a source of *Salmonella* infections (2, 25). The incidence of *Salmonella* in poultry samples varies from 0% to 100% depending on sample sources, sampling and isolation methods (2, 9, 22, 25, 27). In order to minimize the risks of salmonellosis for consumers, microbiological control of the food chain is being increasingly emphasized (20). Thus, the availability of a reliable, rapid *Salmonella* isolation system has become increasingly important for the agricultural and poultry industry, as well as for legislative regulation of food safety.

The ideal *Salmonella* detection method should be sensitive, specific, simple and inexpensive. The methods described in the USDA/FSIS Microbiology Laboratory Guidebook and the FDA/BAM are considered the standard *Salmonella* isolation methods. A 24 h pre-enrichment step in buffered peptone water (BPW) at 35 °C is employed in both methods followed by a 24 h selective enrichment step in tetrathionate (TT) and Rappaport-Vassiliadis (RV) broth. Three different selective agar plates are applied in each method after selective enrichment. In addition to these methods, there are many other *Salmonella* isolation methods available (3, 6, 19, 29), but none was designed specifically based on unique characteristics of poultry products. In poultry houses, *Proteus* spp. is one of the main airborne microflora (21). Our preliminary data indicated that more than 90% chicken samples were *Proteus* spp. positive (data not shown). *Proteus* spp. are tetrathionate reductase positive(15, 16), which can grow very well in TT broth. It can also generate H₂S (10) and show the same phenotypes as *Salmonella* on

bismuth sulfite (BS) agar, xylose lysine desoxycholate (XLD) agar, hektoen enteric (HE) agar plates and triple sugar iron (TSI) slant. The total viable count of bacteria in poultry samples were usually between 10^3 - 10^6 CFU/g. With this high concentration of bacteria as starters, cultures become stationary state after 24 h pre-enrichment in BPW. If the media following pre-enrichment step are insufficiently selective for *Salmonella*, bacteria other than *Salmonella* will overgrow on selective plates. Considering the specific characteristics of poultry samples, it is necessary to develop a poultry-specific *Salmonella* isolation method which selects for the growth of *Salmonella* but suppresses the growth of *Proteus* spp. and other microorganisms present in poultry products.

As a newly certified product, there were no reports on *Salmonella* contamination in organic chicken products in the U.S. The purpose of this study was to develop a simple and rapid poultry-specific *Salmonella* isolation method, and evaluate the method using the standard procedures of FDA/BAM and USDA/FSIS manual and testing organic chicken samples.

MATERIALS AND METHODS

Bacteria strains

S. Typhimurium strain 14028S (API 20E profile: 6705752, *invA* gene positive), *S. Enteritidis* strain CHS45 NA^r (resistant to 100 $\mu\text{g ml}^{-1}$ nalidixic acid) and *P. mirabilis* strain (API 20E profile: 0732000, *invA* gene negative) were used in this study. Bacterial cultures were prepared in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, MI) by incubation at 37 °C with shaking at 230 rpm for 16-18 h.

Growth of *Salmonella* in BPW

S. Typhimurium strain 14028S was tested for growth in BPW (Difco). *S. Typhimurium* was inoculated at 10^0 CFU/400ml into BPW and incubated in water bath at 37°C with shaking at 100 rpm/min. The total plate count of bacteria was determined after 0 h, 3 h and 6 h incubation by spreading onto LB agar plates (Difco).

Growth of *S. Typhimurium* and *P. mirabilis* in *Salmonella* selective medias

S. Typhimurium strain 14028s and *P. mirabilis* were tested for growth in different *Salmonella* selective medias. Briefly, a loopful of stationary phase culture was streaked onto BS (Difco) agar, XLD (Difco) agar, HE (Difco) agar, xylose lysine tergitol 4 agar (XLT4; Difco), brilliant green sulfa agar (BGS; contains 0.1% sodium sulfapyridine; Oxoid LTD, Basingstoke, Hampshire, England) and double modified lysine iron agar (DMLIA; Oxoid). After 24 h of incubation at 35°C, the growth of both strains on the selective agar plates was examined.

TT (Difco) and RV (Difco) broth were inoculated at 10^2 CFU/ml of the *S. Typhimurium* and *P. mirabilis* strains and incubated at 42°C with shaking at 100 rpm/min for 24 h. The concentration of the bacteria in the broths was determined on XLD plates.

Competition growth test of *S. Typhimurium* and *P. mirabilis* in TT and RV broth

S. Typhimurium strain 14028s and *P. mirabilis* were mixed and inoculated at 10^2 CFU/ml of each strain in TT and RV broth and incubated at 42°C with shaking at 100 rpm for 24 h, The concentration of the bacteria in the broths was determined on XLD and XLT4 plates.

Modified chicken *Salmonella* isolation method

A modified procedure based on our data, USDA/FSIS and FDA/BAM methods was used to isolate *Salmonella* from raw chicken products. Briefly, organic chicken carcasses were purchased from local grocery stores and each was placed in BPW (see below). After manual mixing, the chicken rinse was incubated in water bath at 37 °C with shaking at 100 rpm for 6 h, 10 ml and 1 ml pre-enriched rinse was transferred to 100 ml TT and 100 ml RV broth, respectively. After incubation at 42 °C for 24 h, one loopful of TT broth was streaked onto XLT4 agar plate, and another loopful of RV broth onto XLD agar plate.

PCR/API 20 E confirmation

The suspect colonies from selective plates were either confirmed by PCR or API 20 E tests. The PCR procedure was modified from method described previously (13, 18). Typhimurium strain 14028S and a *P. mirabilis* strain were used as reference strains in the PCR test. A 284 bp PCR product targeting *invA* gene was amplified by using primers: *invA*139 (5'-GTGAAATTATCGCCACGTTCTGGGCAA-3') and *invA*141 (5'-TCATCGCACCGTCAAAGGAACC-3'). Primers were synthesized by the Life Technologies (Gaithersburg, MD.). Amplification reactions were carried out with 10 µl of boiled bacterial suspensions, 250 µM deoxynucleoside triphosphate, 2.5 mM MgCl₂, 50 pmol of primers, and 1 U of Gold *Taq* polymerase (Perkin-Elmer, Foster City, CA). Distilled water was added to bring the final volume to 50 µl. The PCR cycle included an initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation for 1 min at

94°C, primer annealing for 1 min at 64°C, and extension for 1 min at 72°C, and a final extension at 72°C for 10 min. The reaction products were then analyzed by electrophoresis in 2.0% agarose gels stained with ethidium bromide, visualized under UV light, and recorded by using a gel documentation system (Bio-Rad, Hercules, CA).

PCR negative suspect isolates were further confirmed by API 20E tests. API 20 E tests were carried out following the standard protocol from the manufacturer (BioMérieux sa, France)

Sensitivity for the modified *Salmonella* isolation method

S. Enteritidis strain CHS45 NA^r (resistant to 100 µg ml⁻¹ nalidixic acid) was used in sensitivity evaluation experiments because the NA^r phenotype enabled easy enumeration of *Salmonella* cells in chicken samples. The strain was grown in 5 ml of LB, containing 100 µg ml⁻¹ nalidixic acid (Sigma), at 37°C in 50 ml flasks with rotary agitation (230 rpm) for 16 to 18 h. Stationary phase cells were harvested by centrifugation at 6,500 × *g* for 6 min at room temperature. Cell pellets were washed in 15 ml of saline and re-suspended in saline to an O.D.₆₀₀ of 0.5 (approx. 5 × 10⁸ CFU ml⁻¹). Cell concentrations were determined via plate counts on LB agar plates. Chicken samples were inoculated with the bacterium at 10⁰CFU/sample and 10¹CFU/sample. *Salmonella* was isolated from the inoculated samples using the modified method described above.

Evaluation of the modified rapid *Salmonella* isolation method

The modified method was evaluated using the standard methods in the FDA/BAM and USDA/FSIS manuals and 44 organic chicken samples.

a. Sample collection and preparation Organic chicken carcasses (n=44) of four brands were randomly collected from three retail organic stores in the Washington, D.C. area. Sampling visits were made on every other Monday for three times. On each sampling day, 14-15 prepackaged organic chicken samples were randomly collected and transported on ice to the laboratory. Each sample was aseptically removed and placed in a sterilized plastic bag which contained 400 ml pre-warmed BPW. The bag was shaken manually for 3 min. The rinse was used for *Salmonella* isolation.

b. The FDA/BAM method The organic chicken rinse was incubated at 35 °C for 22-24 h. One milliliter and 0.1 ml pre-enriched rinse were transferred to 10 ml TT and 10 ml RV broth, respectively. TT broth was incubated at 43°C and RV broth at 42°C. After 20-24 h incubation, one loopful of culture from each broth was streaked onto BS, XLD and HE agar. The plates were incubated at 35°C for 20-24 h and examined for the presence of typical *Salmonella* colonies.

c. The USDA/FSIS method The organic chicken rinse solution was incubated at 35°C for 22-24 h. 0.5 ml and 0.1 ml pre-enriched rinse was transferred to 10 ml TT and 10 ml RV broth respectively. After 22-24 h incubation at 42 °C, one loopful of culture from each broth was streaked onto XLT4, BGS and DMLIA agar plates. The plates were incubated at 35°C for 22-24 h and examined for the presence of typical *Salmonella* colonies.

Three presumptive *Salmonella* colonies from each plate were inoculated onto TSI (GENE-TRAK systems, MA) and urea agar slants (Difco). After 24 h of incubation at 35°C, the isolates with typical *Salmonella* phenotypes (Table 4.1) were further confirmed by PCR or API 20E tests.

RESULTS

A rapid method for isolation and identification of *Salmonella* specifically from poultry products was developed based on the standard procedures of the FDA/BAM and USDA/FSIS *Salmonella* isolation manual. The modified method was evaluated using the standard procedures of the FDA/BAM and USDA/FSIS manual and testing 44 organic chicken samples.

BPW was used in the pre-enrichment for *Salmonella* isolation. When *S. Typhimurium* 14028S was inoculated at 10^0 CFU/400 ml of BPW and incubated in water bath at 37°C with shaking (100 rpm), the number of *Salmonella* increased to 10^4 CFU/400ml after 6 h pre-enrichment, suggesting that 6 h pre-enrichment was sufficient for further isolation of *Salmonella*.

Growth of *S. Typhimurium* and *P. mirabilis* on *Salmonella* selective media

Based on preliminary studies, more than 90% of the chicken samples carried *P. mirabilis* that resembles *Salmonella* (data not shown). In order to effectively isolate *Salmonella* from chicken samples, selective medias that favor the growth of *Salmonella* but suppress the growth of *Proteus* need to be identified.

S. Typhimurium and *P. mirabilis* both grew well on XLD, HE, and BS agar plates with identical phenotypes, whereas *S. Typhimurium* grew well and *P. mirabilis* showed poor growth on XLT4, BGS and DMLIR plates.

Table 4.1. Biochemical characteristics of *Salmonella* on TSI and urea slants (1, 7)

Medium	Reaction/enzymes	Results		<i>Salmonella</i>	Percentage of different <i>Salmonella</i> strains showing the reaction*
		Negative	Positive		
TSI**	Acid production from glucose	Butt red	Butt yellow	+	100
	Acid production from lactose or sucrose	Surface red	Surface yellow	-	99
	Gas production	No air bubbles in butt	Air bubbles in butt	+	92
	H ₂ S production	No black color	Black color	+	92
Urea slant	Urease	Yellow	Rose pink-deep cerise	-	99

* These percentages may vary from country to country and from food product to food product.

** TSI stands for triple sugar iron slant.

When the two organisms each was inoculated at 10^2 CFU/ml in TT and RV broth and incubated at 42°C for 24 h, *S. Typhimurium* showed good growth in both broths, but grew faster in TT broth than in RV broth. *P. mirabilis* only grew in TT broth (Figure 4.1).

Competitive growth of *S. Typhimurium* and *P.mirabilis* in TT broth

The mixture of *S. Typhimurium* and *P. mirabilis* was inoculated each at 10^2 CFU/ml in TT broth and incubated at 42 °C with shaking at 100 rpm for 24 h. The mixed culture was plated onto both XLD and XLT4 plates. The number of *P. mirabilis* was 20 times greater than the number of *S. Typhimurium* after 24 h incubation in TT broth (Figure 4.2). The results were repeated for three times.

Evaluation of the modified method

Because of the large presence of *P. mirabilis* in chicken products, a rapid method specifically designed for isolating *Salmonella* in such products was developed based on the growth characteristics of *P. mirabilis* and *Salmonella* on *Salmonella* selective medias. Briefly, chicken rinses were first incubated for 6 h, instead of 22-24 h, in BPW. After pre-enrichment, 10 or 1 ml culture, instead of 1 or 0.1 ml, were transferred to 100 ml TT or RV broth for 22-24 h selective enrichment respectively.

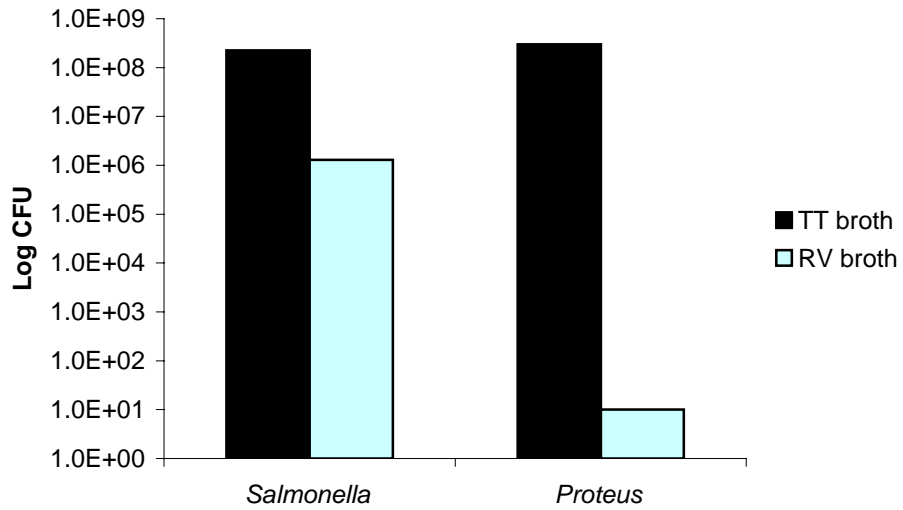


Figure 4.1. Growth of *S. Typhimurium* and *P. mirabilis* in TT and RV broth. *S. Typhimurium* strain 14028s and one *P. mirabilis* strain were inoculated in TT and RV broth at 10^2 CFU/ml respectively and incubated at 42°C with shaking at 100 rpm/min for 24 h.

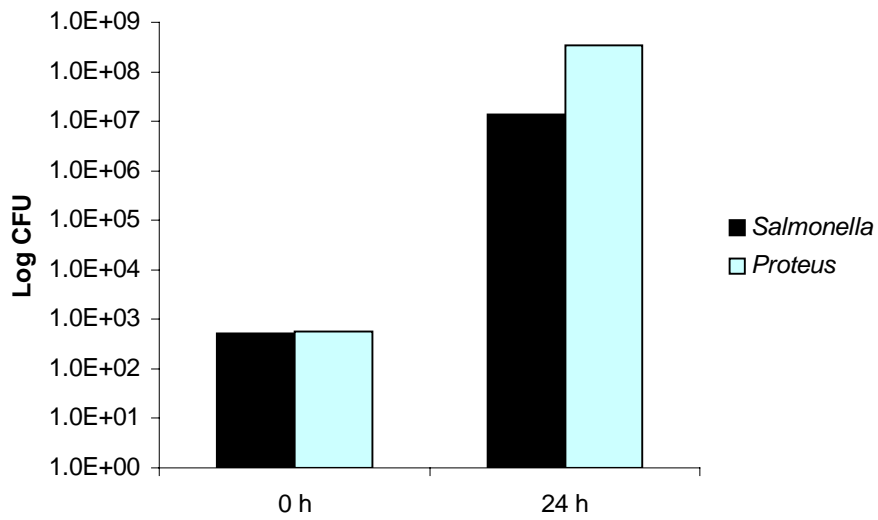


Figure 4.2. Competition test of *S. Typhimurium* and *P. mirabilis* in TT broth. *S. Typhimurium* strain 14028s and *P. mirabilis* were mixed and inoculated at 10^2 CFU/ml of each strain in TT and RV broth and incubated at 42°C with shaking at 100 rpm for 24 h.

One loopful of culture from TT or RV broth was streaked onto XLT4 or XLD plates to examine *Salmonella*-like colonies. When *S. Enteritidis* strain CHS45 was inoculated at 10^0 CFU/sample and 10^1 CFU/sample level, all the inoculated samples were detected by the modified method. The results were repeated for three times. The method was also evaluated by comparing to the USDA/FSIS and FDA standard methods in isolating *Salmonella* from 44 organic chicken samples.

a. *Salmonella* recovery from organic chicken samples after enrichment in TT broth

By following the method in the FDA/BAM, 2% (3/132), 6% (8/132) and 4% (5/129) *Salmonella*-like colonies on XLD, HE, and BS agar plates, respectively, were confirmed as *Salmonella*. The isolation rate of *Salmonella* from organic chicken samples were only 5% (2/44), 11% (5/44) and 7% (3/44) using XLD, HE, and BS agar plate, respectively. Most of the *Salmonella*-like colonies were urease positive and confirmed as *P. mirabilis*.

By following the method in the USDA/FSIS manual, 92% (65/71), 49% (35/72) and 30% (38/125) *Salmonella*-like colonies on XLT4, BGS, and DMLIR agar plates, respectively, were confirmed as *Salmonella*. The isolation rate of *Salmonella* from organic chicken samples were 52% (23/44), 41% (18/44), and 36% (16/44) using XLT4, BGS, and DMLIR agar plates, respectively.

When the modified method was applied (see materials and methods), 100% (81/81) *Salmonella*-like colonies on XLT4 plate were confirmed as *Salmonella*. The isolation rate of *Salmonella* from organic chicken samples was 61% (27/44). (Tables 4.2, 4.4).

b. *Salmonella* recovery from organic chicken samples after enrichment in RV broth

By following the method in the FDA/BAM, 62% (74/120), 70% (82/118) and 56% (72/129) *Salmonella*-like colonies on XLD, HE, and BS agar plates, respectively, were confirmed as *Salmonella*. The isolation rate of *Salmonella* from organic chicken samples were 64% (28/44), 66% (29/44) and 59% (26/44) using XLD, HE, and BS agar plate, respectively.

By following the method in the USDA/FSIS manual, 99% (84/85), 91% (80/88) and 56% (70/126) *Salmonella*-like colonies on XLT4, BGS, and DMLIR agar plates, respectively, were confirmed as *Salmonella*. The isolation rate of *Salmonella* from organic chicken samples were 66% (29/44), 59% (26/44) and 66% (29/44) using XLT4, BGS, and DMLIR agar plates, respectively.

When the modified method was applied, 96% (81/84) *Salmonella*-like colonies on XLD plate were confirmed as *Salmonella*. The isolation rate of *Salmonella* from organic chicken samples was 66% (29/44) (Table 4.3, 4.4).

If the isolation rate of *Salmonella* by using TT and RV broth were merged, the same rate (66%) were found for the FDA/BAM, USDA/FSIS and modified method (Table 4.4).

Table 4.2. *Salmonella* recovery from 44 organic chicken samples by following different methods after enrichment in TT broth

<i>Salmonella</i> isolation methods	Selective Agar	# of tested <i>Salmonella</i> -like colonies ^a	# of colonies confirmed as <i>Salmonella</i> ^b	Specificity for <i>Salmonella</i> ^c (%)
FDA/BAM method	XLD	132	3	2
	HE	132	8	6
	BS	129	5	4
USDA/FSIS method	XLT4	71	65	92
	BGS	72	35	49
	DMLIR	125	38	30
Modified method	XLT4	81	81	100

^a Three *Salmonella*-like colonies were selected from each plate whenever there were suspect colonies.

^b Suspect colonies were confirmed as *Salmonella* either by PCR or API 20 E test.

^c Specificity for *Salmonella* was calculated as the following:

$$\frac{\text{\# of confirmed } Salmonella \text{ colonies by PCR/API 20E}}{\text{\# of total suspect colonies tested}} \times 100\%$$

Table 4.3. *Salmonella* recovery from 44 organic chicken samples by following different methods after enrichment in RV broth

Method	Selective Agar	# of total <i>Salmonella</i> -like colonies ^a	# of colonies confirmed as <i>Salmonella</i> ^b	Specificity for <i>Salmonella</i> ^c (%)
FDA/BAM method	XLD	120	74	62
	HE	118	82	70
	BS	129	72	56
USDA/FSIS method	XLT4	85	84	99
	BGS	88	80	91
	DMLIR	126	70	56
The modified method	XLD	84	81	96

^a Three *Salmonella*-like colonies were selected from each plate whenever there were suspect colonies.

^b Suspect colonies were confirmed as *Salmonella* either by PCR or API 20 E test.

^c Specificity for *Salmonella* was calculated as the following:

$$\frac{\text{\# of confirmed } Salmonella \text{ colonies by PCR/API 20E}}{\text{\# of total suspect colonies tested}} \times 100\%$$

Table 4.4. *Salmonella* recovery rate in 44 organic chicken samples by following different *Salmonella* isolation methods

Methods	Selective Agar	<i>Salmonella</i> recovery rate (%)		
		Enriched in	Enriched in	Total
		TT broth	RV broth	
FDA/BAM method	XLD	5 (2/44)	64 (28/44)	
	HE	11 (5/44)	66 (29/44)	66 (29/44)
	BS	7 (3/44)	59 (26/44)	
USDA/FSIS method	XLT4	52 (23/44)	66 (29/44)	
	BGS	41 (18/44)	66 (29/44)	66 (29/44)
	DMLIR	36 (16/44)	59 (26/44)	
The modified method	XLT4	61 (27/44)	-*	
	XLD	-	66 (29/44)	66 (29/44)

* “-” stands that samples were not tested by using that method.

DISSCUSSION

A reliable, time-saving and low cost method for the isolation and preliminary identification of *Salmonella* from chicken samples was developed based on the FDA/BAM and USDA/FSIS *Salmonella* isolation methods. In general, approximately 95% of the presumptive *Salmonella* colonies that were isolated using the modified method were confirmed as *Salmonella* by PCR/ API 20E tests. This method with high specificity reduced time for isolating *Salmonella* in chicken products by 24-48 hours compared to the USDA and FDA methods. In addition, *Salmonella* isolates recovered using this method may directly be further typed by serotyping and/or PFGE due to its high specificity.

In the USDA/FSIS and FDA/BAM methods, the rinse from chicken samples need to be pre-enriched in BPW for 24 h. After the pre-enrichment, the number of total bacteria reaches as high as 10^9 CFU/ml. Although the number of the *Salmonella* cells also increases, it is usually outnumbered by other bacteria because they have a much higher number of cells when combined. When 1 ml or 0.1 ml pre-enriched sample was transferred into 10 ml TT or RV broth, these bacteria were only 1:10 or 1:100 diluted. Although selective enrichment using TT or RV broth may inhibit the growth of many of these bacteria to a certain extent, their overgrowth likely will mask the growth of *Salmonella*. Consequently, a large number of colonies may appear on selective agar plates, which makes it very difficult to isolate *Salmonella*. Our findings confirmed that non-*Salmonella* colonies covered *Salmonella*-like colonies and that it was impossible to pick single colonies on certain plates.

If samples are pre-enriched in BPW for 6 h, the concentrations of bacterial cells will be significantly lower than those from 24 h incubation. But 6 h pre-enrichment was long enough for one *Salmonella* cell to grow to 10^4 CFU in BPW.

After 6 h pre-enrichment, there will be enough *Salmonella* cells if 1 ml or 10 ml culture is transferred into 100 ml RV or TT selective enrichment broth. Because of the low starting concentration of other bacteria in RV and TT broth, *Salmonella* competes well with other bacteria in selective broths and become the dominant organism when streaked onto a selective agar plate.

A significant difference on specificity and sensitivity between the FDA/BAM and USDA/FSIS methods was found if TT broth was used to enrich *Salmonella* from chicken samples. Among 44 organic chicken samples, only 4 samples were confirmed as *Salmonella* positive if the FDA/BAM method was applied, but 27 samples were confirmed as *Salmonella* positive by the USDA/FSIS method. In TT broth, only bacteria with tetrathionate reductase could grow well (12). But in chicken samples, in addition to *Salmonella*, *Proteus* spp, the major airborne microflora in chicken houses, also possesses tetrathionate reductase(15, 16, 17, 21). They can grow as well as *Salmonella* in TT broth, generating H₂S (10) and showing the same phenotypes as *Salmonella* on XLD, HE, BS plates and TSI slants. But almost all the *Proteus* spp. are urease positive (4, 5) and show poor growth on all three USDA/FSIS recommended selective agar plates. However, when XLD, HE and BS agars were applied after enrichment in RV broth, the specificity and sensitivity were as good as the USDA/FSIS recommended agar plates, because RV broth is more selective than TT broth (14, 26) and *Proteus* spp. grew poorly in RV broth (Figure 4.1).

Although a single selective enrichment medium plus a single selective agar plate work very well in isolating *Salmonella* in chickens, it is important to include additional selective enrichment medium than RV broth as it is a very selective medium and may not propagate certain *Salmonella* strains. In the modified method, two different enrichment broth systems were included. By using this simplified

method, the same positive results were obtained from 44 organic chicken samples (66%) compared with the FDA/BAM and USDA/FSIS methods. The modified method also offers a high specificity compared to the standard methods. Because of its high specificity, the modified method also reduced *Salmonella* isolation and identification procedure to 48 h compared to 3-5 days using standard methods.

More than 66% (29/44) retail organic chicken samples were confirmed *Salmonella* positive. This contamination rate is much higher than that of the conventional retail chicken samples (30). Retail organic chicken products were usually produced by small slaughterhouses. Some research showed *Salmonella* contamination rate was related with the size of slaughterhouses, small slaughterhouses (fewer than 200 birds slaughtered per day) had a much higher *Salmonella* contamination possibility compared with bigger slaughterhouse because of the poor hygienic conditions (8). The widespread occurrence of *Salmonella* in organic chicken samples emphasizes the need for implementation of effective control measures of microbial contaminations in organic poultry products.

REFERENCES

1. Microbiology - General guidance on methods for the detection of *Salmonella* spp., 3rd edition. International Organization for Standardization, Geneva, Switzerland. 679, 1993.
2. **Antunes, P., C. Reu, J. C. Sousa, L. Peixe, and N. Pestana.** 2003. Incidence of *Salmonella* from poultry products and their susceptibility to antimicrobial agents. *Int J Food Microbiol* **82**:97-103.
3. **Blivet, D., G. Salvat, F. Humbert, and P. Colin.** 1997. Evaluation of a new enrichment broth for the isolation of *Salmonella* spp. from poultry products. *Int J Food Microbiol* **38**:211-6.

4. **Coker, C., O. O. Bakare, and H. L. Mobley.** 2000. H-NS is a repressor of the *Proteus mirabilis* urease transcriptional activator gene ureR. J Bacteriol **182**:2649-53.
5. **Dattelbaum, J. D., C. V. Lockett, D. E. Johnson, and H. L. Mobley.** 2003. UreR, the transcriptional activator of the *Proteus mirabilis* urease gene cluster, is required for urease activity and virulence in experimental urinary tract infections. Infect Immun **71**:1026-30.
6. **Fung, D. Y., and A. A. Kraft.** 1970. A rapid and simple method for the detection and isolation of *Salmonella* from mixed cultures and poultry products. Poult Sci **49**:46-54.
7. **Funk, J. A., P. R. Davies, and M. A. Nichols.** 2000. The effect of fecal sample weight on detection of *Salmonella enterica* in swine feces. J Vet Diagn Invest **12**:412-8.
8. **Fuzihara, T. O., S. A. Fernandes, and B. D. Franco.** 2000. Prevalence and dissemination of *Salmonella* serotypes along the slaughtering process in Brazilian small poultry slaughterhouses. J Food Prot **63**:1749-53.
9. **Jorgensen, F., R. Bailey, S. Williams, P. Henderson, D. R. Wareing, F. J. Bolton, J. A. Frost, L. Ward, and T. J. Humphrey.** 2002. Prevalence and numbers of *Salmonella* and *Campylobacter* spp. on raw, whole chickens in relation to sampling methods. Int J Food Microbiol **76**:151-64.
10. **Kalina, G. P.** 1983. [Combined media for the simplified and rapid identification of bacteria of the *Proteus-Providencia* group]. Zh Mikrobiol Epidemiol Immunobiol:53-7.
11. **Karras, D. J.** 2000. Incidence of foodborne illnesses: preliminary data from the foodborne diseases active surveillance network (FoodNet). Ann Emerg Med **35**:92-3.
12. **Knox, R., P. H. Gell, and M. R. Pollack.** 1942. Selective media for organisms of the *Salmonella* group. J. Pathol. Bacteriol. **54**:469-483.
13. **Malorny, B., J. Hoorfar, C. Bunge, and R. Helmuth.** 2003. Multicenter Validation of the Analytical Accuracy of *Salmonella* PCR: towards an International Standard. Appl Environ Microbiol **69**:290-6.
14. **Munoz, A., E. Frapolli, M. A. Morinigo, J. J. Borrego, and P. Romero.** 1987. Isolation of *Salmonella* using three selective enrichment broths from sausages naturally contaminated. Its relationship with other microorganisms that produce food toxi-infections. Zentralbl Bakteriologie Mikrobiologie Hygiene [B] **185**:105-11.
15. **Oltmann, L. F., V. P. Claassen, P. Kastelein, W. N. Reijnders, and A. H. Stouthamer.** 1979. Influence of tungstate on the formation and activities of four reductases in *Proteus mirabilis*: identification of two new molybdo-enzymes: chlorate reductase and tetrathionate reductase. FEBS Lett **106**:43-6.

16. **Oltmann, L. F., and A. H. Stouthamer.** 1975. Reduction of tetrathionate, trithionate and thiosulphate, and oxidation of sulphide in proteus mirabilis. Arch Microbiol **105**:135-42.
17. **Papavassiliou, J., V. Samaraki-Lyberopoulou, and G. Piperakis.** 1969. Production of tetrathionate reductase by *Salmonella*. Can J Microbiol **15**:238-40.
18. **Rahn, K., S. A. De Grandis, R. C. Clarke, S. A. McEwen, J. E. Galan, C. Ginocchio, R. Curtiss, 3rd, and C. L. Gyles.** 1992. Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. Mol Cell Probes **6**:271-9.
19. **Read, S. C., R. J. Irwin, C. Poppe, and J. Harris.** 1994. A comparison of two methods for isolation of *Salmonella* from poultry litter samples. Poult Sci **73**:1617-21.
20. **Rose, B. E., W. E. Hill, R. Umholtz, G. M. Ransom, and W. O. James.** 2002. Testing for *Salmonella* in raw meat and poultry products collected at federally inspected establishments in the United States, 1998 through 2000. J Food Prot **65**:937-47.
21. **Sauter, E. A., C. F. Petersen, E. E. Steele, J. F. Parkinson, J. E. Dixon, and R. C. Stroh.** 1981. The airborne microflora of poultry houses. Poult Sci **60**:569-74.
22. **Sofos, J. N., S. L. Kochevar, J. O. Reagan, and G. C. Smith.** 1999. Incidence of *Salmonella* on beef carcasses relating to the U.S. meat and poultry inspection regulations. J Food Prot **62**:467-73.
23. **Tirado, C., and K. Schmidt.** 2001. WHO surveillance programme for control of foodborne infections and intoxications: preliminary results and trends across greater Europe. World Health Organization. J Infect **43**:80-4.
24. **Trejejo, R. T., J. G. Courtney, M. Starr, and D. J. Vugia.** 2003. Epidemiology of salmonellosis in California, 1990-1999: morbidity, mortality, and hospitalization costs. Am J Epidemiol **157**:48-57.
25. **Uyttendaele, M., P. De Troy, and J. Debevere.** 1999. Incidence of *Salmonella*, *Campylobacter jejuni*, *Campylobacter coli*, and *Listeria monocytogenes* in poultry carcasses and different types of poultry products for sale on the Belgian retail market. J Food Prot **62**:735-40.
26. **Vassiliadis, P., C. Mavromati, D. Trichopoulos, V. Kalapothaki, and J. Papadakis.** 1987. Comparison of procedures based upon Rappaport-Vassiliadis medium with those using Muller-Kauffmann medium containing Teepol for the isolation of *Salmonella sp.* Epidemiol Infect **99**:143-7.

27. **Veldman, A., H. A. Vahl, G. J. Borggreve, and D. C. Fuller.** 1995. A survey of the incidence of *Salmonella* species and *Enterobacteriaceae* in poultry feeds and feed components. *Vet Rec* **136**:169-72.
28. **Wallace, D. J., T. Van Gilder, S. Shallow, T. Fiorentino, S. D. Segler, K. E. Smith, B. Shiferaw, R. Etzel, W. E. Garthright, and F. J. Angulo.** 2000. Incidence of foodborne illnesses reported by the foodborne diseases active surveillance network (FoodNet)-1997. FoodNet Working Group. *J Food Prot* **63**:807-9.
29. **Waltman, W. D., and E. T. Mallinson.** 1995. Isolation of *Salmonella* from poultry tissue and environmental samples: a nationwide survey. *Avian Dis* **39**:45-54.
30. **Zhao, C., B. Ge, J. De Villena, R. Sudler, E. Yeh, S. Zhao, D. G. White, D. Wagner, and J. Meng.** 2001. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D.C., area. *Appl Environ Microbiol* **67**:5431-6.

Chapter 5 Prevalence and Characterization of *Salmonella* Serovars from Retail Organic and Conventional Chicken Products

ABSTRACT

The number of organic livestock produced under certified organic farming systems has increased sharply during the last decade. The market for organic food products is expected to grow considerably now that organic labeling is permitted by the USDA. However, there is a paucity of data with regards to the microbiological status of organic poultry products. The objective of this study was to determine the prevalence of *Salmonella* in retail organically produced chicken products. *Salmonella* isolates were characterized by serotyping, pulsed-field gel electrophoresis (PFGE) and antimicrobial-susceptibility testing. Among 198 organic chicken samples, 121 (61%) were *Salmonella* positive, compared to 27 (44%) *Salmonella* positive among 61 conventional chicken samples. Kentucky, Heidelberg, and Typhimurium were top serotypes identified and accounted for more than 60% of all *Salmonella* isolates from chicken samples. There were 37 PFGE patterns identified among isolates from 148 chicken samples, of which 14 patterns were found only in conventional chicken samples, 18 patterns were found only in organic chicken samples, and 5 patterns were shared by both conventional and organic chicken samples. Antimicrobial-susceptibility testing results showed that antimicrobial resistance in *Salmonella* was associated with serotypes. All *S.* Typhimurium positive conventional chicken samples contained isolates that were resistant to at least five antimicrobials. But antibiotic-resistant Typhimurium isolates were only recovered in 21% of organic chicken samples which were resistant to maximum 4 different antimicrobials. The similar trend was found among other serotypes. Our results indicate that organic

chicken samples were frequently contaminated with *Salmonella*, and that *Salmonella* from organic chicken were more susceptible to antimicrobials commonly used in human and veterinary medicine.

INTRODUCTION

In October 2002, the USDA established criteria for organic food (2) which detailed the methods, practices and substances used in producing and handling organic crops and livestock. It also established clear organic labeling criteria (21) and any food meeting the criteria can carry a “USDA Organic” seal. Consumers have been increasingly interested in organic food products. Over the past decade the market for organic food has grown by 20-25% each year, which is five times faster than food sales in general (13). However, there is a paucity of data with regards to the microbiological safety of organic animal products in the United States. There is a greater risk of contamination of organic animal products with foodborne pathogens compared to conventionally raised food animals due to outdoor production and prohibition of antibiotic uses in organic livestock production.

Furthermore, the use of antibiotics for growth promotion, disease treatment and prevention in conventional food animal production has been linked to the development of antibiotic resistance in bacteria (11, 12, 27). Animal drugs, including hormones and antibiotics, are prohibited from organic feed in the Production Standards for Organic Animal Product (4), bacteria in organic animal products are likely more susceptible to antibiotics and this may decrease the development and dissemination of antibiotic-resistant pathogens, thereby prolong the effectiveness of antibiotics for disease treatment in human beings and animals.

Salmonella can colonize in the gastrointestinal tracts without causing any clinical symptoms in a wide range of wild and domestic animals, including animals raised for human consumption. Numerous epidemiological studies have identified food of animal origin as the major vehicles associated with salmonellosis. Contaminated raw or undercooked poultry products are particularly important in transmitting *Salmonella* (10, 15). Nontyphoid *Salmonella* was also selected as the sentinel organism in the National Antimicrobial Resistance Monitoring System (NARMS) to monitor changes in antimicrobial susceptibilities of zootomic pathogens (20).

In this chapter, more than two hundred organic and conventional whole chicken samples were collected from local supermarkets of the Washington, D.C. area. The prevalence of *Salmonella* in organic and conventional chicken samples was determined and isolates were characterized by serotyping, pulsed-field gel electrophoresis (PFGE) and antibiotics susceptibility testing. The results were compared between organic and conventional chicken samples.

MATERIALS AND METHODS

Samples collection and preparation

Four brands of organic chicken carcasses and several brands of conventional chicken carcasses were randomly collected from 3 organic stores and 2 conventional stores in the Washington, D.C. area. Sampling visits were made on every other Monday for seven months for organic and 3 months for conventional chicken samples. On each sampling day, 8-15 prepackaged organic and 8-12 conventional chicken samples were selected and transported on ice to the laboratory. Each sample was aseptically removed and placed in a sterilized plastic bag that contained 400 ml

pre-warmed buffered peptone water (BPW, Difco Laboratories, Detroit, MI.). The bag was shaken manually for 3 min. The rinse solution was used for *Salmonella* isolation.

***Salmonella* isolation method**

A modified method based on USDA Food Safety and Inspection Service (FSIS) Microbiology Laboratory Guidebook was used to isolate *Salmonella* (5). Briefly, the chicken rinse was incubated in 37 °C water bath with shaking at 100 rpm/min for 6 h, and 10 ml pre-enriched rinse was transferred to 100 ml tetrathionate (TT, Difco) broth and incubated at 42 °C with shaking at 100 rpm/min for 24 h. After incubation, one loopful of TT broth was streaked onto xylose lysine tergitol 4 (XLT4, Difco) agar plate.

Three colonies of presumptive *Salmonella* on XLT4 plate were picked and streaked onto XLT4 plates for purification. The purified isolates were inoculated onto triple sugar iron (TSI; GENE-TRAK systems, MA) and urea agar slants (Difco). After 24 h of incubation at 35 °C, the isolates with typical *Salmonella* phenotypes were confirmed by PCR or API 20E.

***Salmonella* PCR confirmation**

A PCR procedure described previously was used (19, 23). Typhimurium strain 14028S and a *Proteus mirabilis* strain were used as reference strains in the PCR test. A 284 bp PCR product targeting *invA* gene was amplified by using primers: *invA*139 (5'-GTGAAATTATCGCCACGTTTCGGGCAA-3') and *invA*141 (5'-TCATCGCACCGTCAAAGGAACC-3'). Primers were synthesized by the Life Technologies (Gaithersburg, MD.). Amplification reactions were carried out with

10 μ l of boiled bacterial suspensions, 250 μ M deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 50 pmol of primers, and 1 U of Gold *Taq* polymerase (Perkin-Elmer, Foster City, CA). Distilled water was added to bring the final volume to 50 μ l. The PCR cycle included an initial denaturation at 94°C for 10 min, followed by 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 64°C, and extension for 1 min at 72°C, and a final extension at 72°C for 10 min. The reaction products were then analyzed by electrophoresis in 2.0% agarose gels stained with ethidium bromide, visualized under UV light, and recorded by using a gel documentation system (Bio-Rad, Hercules, CA).

PFGE

PFGE was performed to determine DNA fingerprinting profiles of organic and conventional chicken *Salmonella* isolates. The PFGE procedure of the Centers for Disease Control and Prevention was used (3, 7, 26). Briefly, bacteria were grown on TSA plates supplemented with 5% defibrinated sheep blood (Becton Dickinson Microbiology Systems, Cockeysville, MD.) at 37°C for 18 h. Bacterial colonies were suspended in cell suspension buffer (100 mM Tris HCl, 100 mM EDTA; pH 8.0) and adjusted to an optical density of 0.50 to 0.54 using Dade MicroScan Turbidity Meter (Dade Behring, Inc., West Sacramento, CA). The cell suspension (200 μ l) was mixed with 10 μ l of proteinase K (10 mg/ml) and an equal volume of melted 1% SeaKem Gold agarose (FMC BioProducts, Rockville, Maine) containing 1% sodium dodecyl sulfate. The mixture was carefully dispensed into a sample mold (Bio-Rad). After solidification, the plugs were transferred to a tube containing 5 ml of lysis buffer (50 mM Tris HCl; 50 mM EDTA, pH 8.0; 1% sarcosyl) and 0.1 mg of proteinase K

per ml. Cells were lysed overnight in a water bath at 54°C with agitation at 180 rpm. After lysis, the plugs were washed twice with distilled water and four times with TE buffer (10 mM Tris HCl, 1 mM EDTA; pH 8.0) for 15 min per wash at 50°C with agitation at 180 rpm. Agarose-embedded DNA was digested with 50 U of *Xba*I (Boehringer Mannheim) overnight in a water bath at 37°C. The plugs were placed in a 1% SeaKem Gold agarose gel. Restriction fragments were separated by electrophoresis in 0.5× TBE (Tris-borate-EDTA) buffer supplemented with 50 µM thiourea (9, 17, 24, 25) at 14°C for 18 h using a Chef Mapper (Bio-Rad) with pulse times of 2.16 to 54.17 s. *S. Braenderup* H9812 was included on each gel as standard. The gel was stained with ethidium bromide, and DNA bands were visualized with a UV transilluminator.

***Salmonella* serotyping**

Salmonella serotypes were determined at the National Veterinary Service Laboratories of the Department of Agriculture in Ames, Iowa by following the standard protocol.

Antimicrobial minimal inhibitory concentration (MIC) test

Antimicrobial MICs of *Salmonella* isolates were determined via the Sensititre automated antimicrobial susceptibility system (Trek Diagnostic Systems, Westlake, Ohio) and interpreted according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines for broth microdilution methods (22). Sensititre susceptibility testing was performed according to the manufacturer's instructions. The following antimicrobials were tested: amikacin, amoxicillin-clavulanic acid, ampicillin, apramycin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol,

ciprofloxacin, florfenicol, gentamicin, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim-sulfamethoxazole. *E. coli* ATCC 25922 and 35218, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853 were used as controls in antimicrobial MIC determinations.

RESULTS

Prevalence of *Salmonella* in organic and conventional chicken samples

A total of 259 (198 organic and 61 conventional) chicken carcasses randomly collected from 5 retail stores were tested for the presence of *Salmonella*. More than 60% (121/198) of the organic chicken samples were found *Salmonella* positive compared to 44% (27/61) among the conventional chicken samples (Table 5.1).

The chicken samples belonged to nine producers (four organic and five conventional). There was a significant difference in the *Salmonella* positive rates among different chicken brands. The *Salmonella* positive rates of four organic chicken brands were 71%, 62%, 54% and 50%, respectively, whereas the rate of one dominant conventional chicken brand was 49%, and 39% samples from the other brands of the conventional chickens was positive for *Salmonella* (Table 5.1).

Serotypes

Eleven serotypes were identified from 121 organic chicken samples. Kentucky and Heidelberg were major serotypes identified in organic chicken samples. Typhimurium was mainly found in two of the organic chicken brands. Other serotypes identified in the organic chickens include Agona, Istanbul, Montevideo, Ohio, Braenderup, Worthington, Thompson and Newport. Among 21 organic chicken

samples, two serotypes were recovered simultaneously from a single sample. Seven organic chicken samples contained Typhimurium and Kentucky, 10 had Heidelberg and Kentucky. Other combinations included Heidelberg-Ohio, Kentucky-Braenderup, Heidelberg-Thompson and Kentucky-Worthington (Table 5.2).

Eight serotypes were identified from 27 conventional whole chicken samples. Typhimurium and Kentucky were predominant serotypes among the conventional chicken samples. Other serotypes included Heidelberg, Istanbul, Montevideo, Enteritidis, Thompson and O4:H12 nonmotile. In three conventional chicken samples, two serotypes were found from each sample: Typhimurium and Kentucky were found in two samples, and Typhimurium and Istanbul were found in one sample (Table 5.2).

Table 5.1. *Salmonella* recovery from organically and conventionally produced chicken products

	Organic chicken ^a					Conventional chicken ^a		
	O1	O2	O3	O4	Total	C1	Other brands	Total
Rate ^b (%)	62 (16/26)	71 (58/82)	54 (29/54)	50 (18/36)	61 (121/198)	49 (20/41)	39 (7/18)	44 (27/61)

^a O1-O4: four organically produced chicken brands; C1: conventionally produced chicken brand; other brands: other conventionally produced chicken brands

^b Rate: recovery rate of *Salmonella* from tested chicken products

Table 5.2. Occurrence of *S. enterica* serotypes among organically and conventionally produced chicken samples

<i>S. enterica</i> serotype	Percentage of <i>Salmonella enterica</i> serotypes among different chicken brands								
	O1 ^a	O2	O3	O4	Total	C1 ^a	Other conventional chicken brands	Total	
Kentucky	56 (9/16)	57 (33/58)	79 (23/29)	39 (7/18)	60 (72/121)	40 (8/20)	29 (2/7)	37 (10/27)	
Heidelberg	31 (5/16)	48 (28/58)	21 (6/29)	11 (2/18)	33 (40/121)	–	14 (1/7)	4 (1/27)	
Typhimurium	6 (1/16)	16 (9/58)	3 (1/29)	44 (8/18)	17 (20/121)	60 (12/20)	–	44 (12/27)	
Istanbul	6 (1/16)	– ^b	–	6 (1/18)	2 (2/121)	5 (1/20)	–	4 (1/27)	
Montevid	–	–	–	6 (1/18)	1 (1/121)	–	14 (1/7)	4 (1/27)	

Table 5.2 (continued)

Thompson	–	2 (1/58)	–	–	1 (1/121)	–	29 (2/7)	7 (2/27)
Ohio	–	5 (3/58)	–	–	3 (3/121)	–	–	–
Agona	–	–	3 (1/29)	–	1 (1/121)	–	–	–
Braenderup	–	–	3 (1/29)	–	1 (1/121)	–	–	–
Newport	–	–	–	6 (1/18)	1 (1/121)	–	–	–
Worthington	6 (1/16)	–	–	–	1 (1/121)	–	–	–

Table 5.2 (continued)

O4:H12	-	-	-	-	-	5	-	4
Nonmotile						(1/20)		(1/27)
Enteritidis	-	-	-	-	-	-	14	4
							(1/7)	(1/27)

^a O1-O4: four organically produced chicken brands; C1: conventionally produced chicken brand; other brands: other conventionally produced chicken brands

^b “-“ stands for no sample was found for that serotype.

PFGE

All 429 *Salmonella* isolates (355 isolates from 121 organic and 74 isolates from 27 conventional chicken samples) were analyzed for their genetic relatedness using PFGE (Figure 5.1). Thirty-seven distinct PFGE patterns were identified, including three main clusters that were constituted by *S. Kentucky*, *S. Heidelberg* and *S. Typhimurium*. Five main PFGE patterns were identified among serotypes Kentucky, Heidelberg and Typhimurium. Among the 37 PFGE patterns, 14 were found only in conventional chicken samples, whereas 18 were identified only in organic chicken samples and 5 were recognized in both conventional and organic samples. From 24 organic and 4 conventional chicken samples, two different PFGE patterns were identified in *Salmonella* isolates from the same samples. Eleven and four PFGE patterns were identified in *S. Typhimurium* isolated from conventional and organic chicken samples, respectively. Six PFGE patterns were identified from *S. Kentucky* isolates: three patterns in *S. Kentucky* from organic chicken samples and the other three patterns in *S. Kentucky* from both organic and conventional chicken samples.

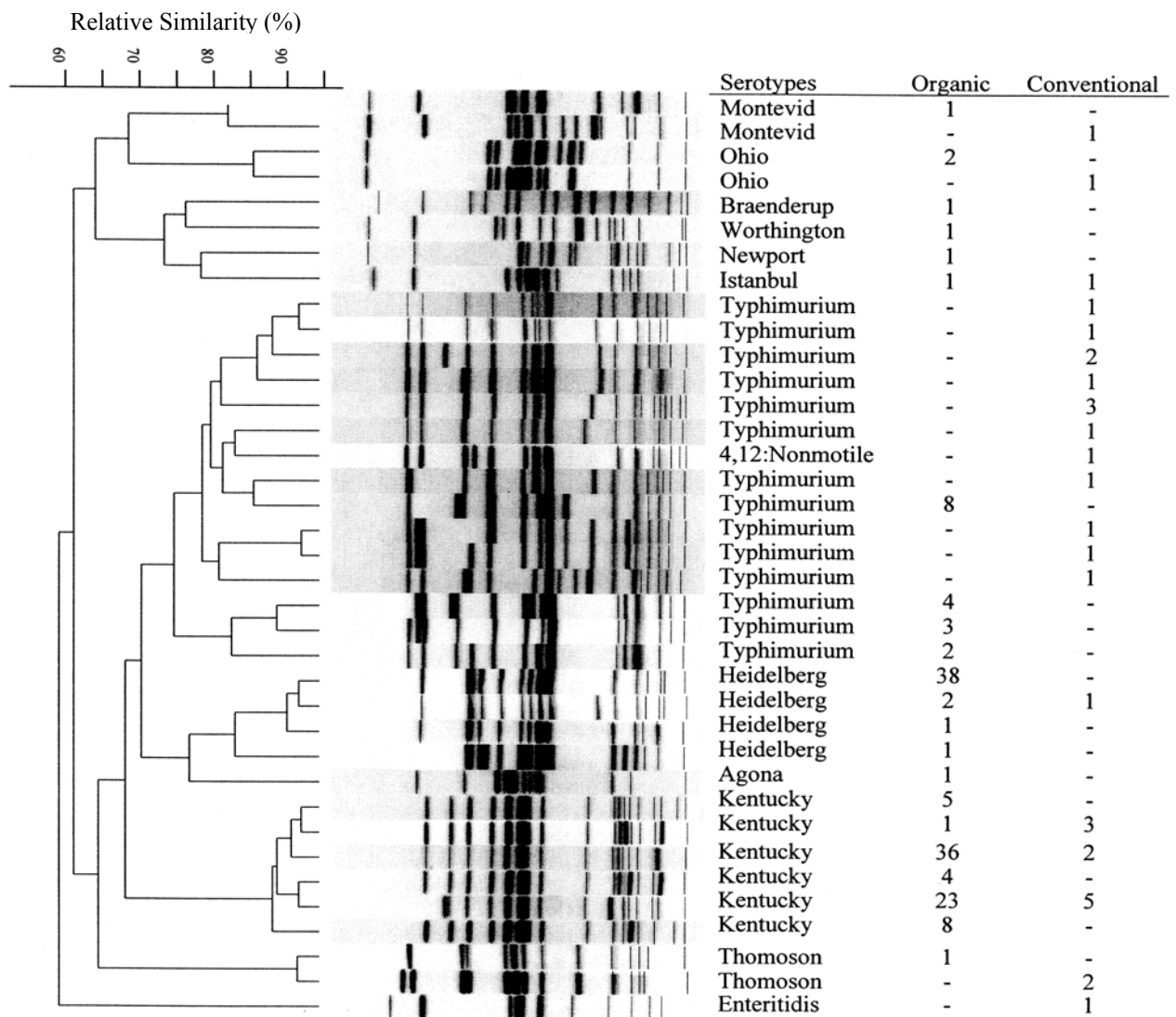


Figure 5.1 Dendrogram of patterns obtained by PFGE of *Salmonella enterica* isolates recovered from different brands of organically and conventionally produced chicken samples

a “-” stands no sample was found for that pattern.

b Restriction enzyme *XbaI* was used in the PFGE protocol.

c The tree indicating genetic similarity was constructed on the basis of the neighbor-joining method. Values of 100 percent mean that the strains are identical.

Antimicrobial susceptibility

Minimum inhibitory concentration (MIC) of 15 antibiotics (Table 5.3) was determined for the 429 *Salmonella* isolates (74 isolates from 27 conventional and 355 isolates from 121 organic chicken samples).

S. Kentucky isolates were identified from 72 organic and 10 conventional chicken samples. Different antibiotic resistance profiles were identified among these isolates from the two sources. *S. Kentucky* from conventional chicken samples were more likely resistant to penicillins and cephalosporins than those from the organic chicken samples (Table 5.3). Most organic and conventional chicken samples contained *Kentucky* isolates either resistant to tetracycline and streptomycin or susceptible to all 15 antibiotics tested. There were a few organic and conventional chicken samples which had isolates that were resistant to 3 to 7 different antibiotics (Table 5.4).

S. Typhimurium isolates were recovered from 12 conventional and 19 organic chicken samples. All 12 conventional chicken samples contained isolates that were resistant to 5-7 different antibiotics. In contrast, *S. Typhimurium* isolates recovered from 79% of the organic chicken samples were susceptible to all 15 antibiotics tested, and antibiotic-resistant *S. Typhimurium* isolates were recovered only from 4 organic chicken samples and these isolates were resistant to 1-4 different antibiotics (Table 5.5).

S. Heidelberg isolates were recovered from 40 organic and 1 conventional chicken samples. Isolates from 75% of the organic chicken samples were susceptible to all 15 antibiotics tested. However, several organic chicken isolates were resistant to up to 9 antibiotics (Table 5.6).

Table 5.3. Occurrence of antimicrobial-resistant Kentucky and Typhimurium isolates among conventionally and organically produced chicken samples

Antimicrobial agent	MIC ($\mu\text{g/ml}$) ^{a,b}	Kentucky		Typhimurium	
		Organic	Conventional	Organic	Conventional
Phenicol					
Chloramphenicol (CHL)	≥ 32	0/72 (0%) ^c	0/10 (0%) ^c	0/19 (0%) ^c	0/12 (0%) ^c
Penicillins					
Amoxicillin-clavulanic acid (AUG)	≥ 32	3/72 (4%)	2/10 (20%)	0/19 (0%)	12/12 (100%)
Ampicillin (AMP)	≥ 32	3/72 (4%)	2/10 (20%)	0/19 (0%)	12/12 (100%)
Cephalosporins					
Cephalothin (CEP)	≥ 32	3/72 (4%)	2/10 (20%)	0/19 (0%)	12/12 (100%)
Ceftiofur (TIO)	≥ 8	3/72 (4%)	2/10 (20%)	0/19 (0%)	12/12 (100%)
Ceftriaxone (AXO)	≥ 64	0/72 (0%)	0/10 (0%)	0/19 (0%)	0/12 (0%)

Table 5.3 continued

Cefoxitin (FOX)	≥32	3/72 (4%)	2/10 (20%)	0/19 (0%)	12/12 (100%)
Tetracycline (TET)	≥16	48/72 (67%)	8/10 (80%)	3/19 (16%)	5/12 (41%)
Aminoglycosides					
Amikacin (AMI)	≥64	0/72 (0%)	0/10 (0%)	0/19 (0%)	0/12 (0%)
Gentamicin (GEN)	≥16	5/72 (7%)	0/10 (0%)	1/19 (5%)	0/12 (0%)
Kanamycin (KAN)	≥64	1/72 (1%)	0/10 (0%)	1/19 (5%)	0/12 (0%)
Streptomycin (STR)	≥64	48/72 (67%)	6/10 (60%)	2/19 (11%)	3/12 (25%)
Sulfonamides and potentiated sulfonamides					
Sulfamethoxazole (SMX)	≥512	5/72 (7%)	0/10 (0%)	1/19 (5%)	0/12 (0%)
Trimethoprim/Sulfamethoxazole (COT)	≥4	0/72 (0%)	0/10 (0%)	0/19 (0%)	0/12 (0%)

Table 5.3 continued

Quinolones and fluoroquinolones					
Nalidixic acid (NAL)	≥32	0/72 (0%)	0/10 (0%)	2/19 (11%)	0/12 (0%)
Ciprofloxacin (CIP)	≥4	0/72 (0%)	0/10 (0%)	0/19 (0%)	0/12 (0%)
Sensitive to all antibiotics		23/72 (32%)	2/10 (20%)	15/19 (79%)	0/12 (0%)

^aAntimicrobial susceptibility testing was performed according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines. *Escherichia coli* (ATCC 25922 and ATCC 35218), *Enterococcus faecalis* (ATCC 51299), and *Pseudomonas aeruginosa* (ATCC 27853) were used as quality controls.

^b“MIC” stands for minimal inhibition concentration

^c *S. Kentucky* was recovered from 72 organically produced and 10 conventionally produced chicken samples; *S. Typhimurium* was recovered from 19 organically produced and 12 conventionally produced chicken samples.

Table 5.4. Occurrence of antibiotic-resistant *S. Kentucky* among organic and conventional chicken samples containing *S. Kentucky*

Antibiotic-resistant profile	Percentage of samples containing antibiotic-resistant <i>S. Kentucky</i> isolates	
	Organic	Conventional
AUG-AMP-CEP-TIO-FOX-TET-STR	3% (2/72)*	0% (0/10)*
AUG-AMP-CEP-TIO-FOX-TET	0% (0/72)	10% (1/10)
AUG-AMP-CEP-TIO-FOX	1% (1/72)	10% (1/10)
TET-STR-GEN-SMX	7% (5/72)	0% (0/10)
TET-STR- KAN	1% (1/72)	0% (0/10)
TET-STR	56% (40/72)	60% (6/10)
TET	1% (1/72)	0% (0/10)
Sensitive to all tested antibiotics	32%(23/72)	20% (2/10)

* *S. Kentucky* was recovered from 72 organically produced and 10 conventionally produced chicken samples.

Table 5.5. Occurrence of antibiotic-resistant *S. Typhimurium* among organic and conventional chicken samples containing *S. Typhimurium*

Antibiotic-resistant profiles	Percentage of samples containing antibiotic-resistant isolates	
	Organic	Conventional
AUG-AMP-CEP-TIO-FOX-TET-STR	0% (0/19) *	17% (2/12) *
AUG-AMP-CEP-TIO-FOX-TET	0% (0/19)	17% (2/12)
AUG-AMP-CEP-TIO-FOX	0% (0/19)	67% (8/12)
TET-STR-GEN-SMX	5% (1/19)	0% (0/12)
TET-STR	5% (1/19)	8% (1/12)
NAL-KAN	5% (1/19)	0% (0/12)
NAL	5% (1/19)	0% (0/12)
Sensitive to all tested antibiotics	79% (15/19)	0% (0/12)

* *S. Typhimurium* was recovered from 19 organically produced and 12 conventionally produced chicken samples.

Table 5.6. Occurrence of antibiotic-resistant *S. Heidelberg* among organic and conventional chicken samples containing *S. Heidelberg*

Antibiotic-resistant profiles	Percentage of samples containing antibiotic-resistant isolates	
	Organic	Conventional
CHL-AUG-AMP-CEP-TIO- FOX-TET-STR-SMX	3% (1/40) *	0% (0/1)
TET-SMX-STR-GEN	3% (1/40)	0% (0/1)
AMP-CEP	5% (2/40)	0% (0/1)
TET-STR	5% (2/40)	100% (1/1) *
TET	8% (3/40)	0% (0/1)
FOX	3% (1/40)	0% (0/1)
Sensitive to all antibiotics	75% (30/40)	0% (0/1)

* *S. Heidelberg* was recovered from 40 organically produced and 1 conventionally produced chicken samples.

Table 5.7. Occurrence of minority antibiotic-resistant *S. enterica* serotypes among organic and conventional chicken samples containing minority *S. enterica* serotypes

<i>S. enterica</i> serotype	Antibiotic-resistant profile	# of samples contained antibiotic-resistant isolates	
		Organic	Conventional
Newport	CHL-AUG-AMP-CEP-TIO- FOX-TET-STR-SMX	1	0
4,12:Nonmotile	AUG-AMP-CEP-TIO-FOX	0	1
Enteritidis	TET-GEN-STR-SMX	0	1
Istanbul	TET-STR	1	1

Ten other serotypes were also recovered from several organic and conventional chicken samples. Surprisingly, a *S. Newport* isolated from an organic chicken sample was resistant to 9 antibiotics. *S. Istanbul* were identified from one organic and one conventional chicken sample and were resistant to tetracycline and streptomycin. *S. Enteritidis* and *S. 4,12:Nonmotile* were identified from two conventional chicken samples and were resistant to 4 and 5 antibiotics, respectively (Table 5.7). Interestingly, none of the isolates of serotypes Ohio, Agona, Braenderup, Thompson, Worthington and Montevideo was resistant to any of the 15 antibiotics tested.

DISCUSSION

According to the *Salmonella* performance standard 9 CFR 381.94(b) of the US Department Agriculture, a chicken slaughter operation is required to test *Salmonella*. A maximum of 12 *Salmonella* positive samples are allowed in a 51-sample set and plants that exceed 12 positive *Salmonella* samples in the 51-sample set must initiate corrective action to meet the performance standard. In this study, it was found that 61 (121/198) of the organic and 44% (27/61) of the conventional chicken samples contained *Salmonella* which were much higher than 20% from the baseline study of the USDA/FSIS conducted in 1996 and the Broiler Production *Salmonella* Performance Standard. This difference might be due in part to the different methods used for isolating *Salmonella* and the sample sources. The isolation method used in this chapter was modified from the USDA/FSIS *Salmonella* isolation manual, 6 h pre-enrichment in BPW was applied instead of 24 h, which could decrease the starter concentration of non-*salmonella* bacteria in selective broth and increase the selectivity. Data from chapter 4 has shown 6 h pre-

enrichment in BPW was sufficient for one *Salmonella* cell to grow up to 10-100 cells/ml in 400 ml culture. After pre-enrichment, 10 ml samples were transferred into 100 ml TT broth for 24 h selective enrichment and then streaked onto XLT4 plates. Although prevalence of *Salmonella* in chicken samples was high, no samples containing more than 100 *Salmonella* cells were found when MPN was determined for more than 30 organic and conventional chicken samples (data not shown).

Chicken samples tested in this chapter were collected from retail stores which were different from the sample sources in the baseline study of the USDA/FSIS conducted in 1996. Both the baseline study and the Broiler Production *Salmonella* Performance Standard require that samples should be collected from the slaughterhouses. There are more chances for chicken samples to be contaminated with *Salmonella* during storage, shipment and handling in retail stores.

Research from Europe showed that there was no increase in *Salmonella* positive rate in broilers, swine, pork, and chicken meat after the use of antimicrobial growth promoters were discontinued (14). But in our research, the prevalence of *Salmonella* in organic chicken samples was significantly higher than conventional chicken samples. Several reasons may have caused this difference. First of all, organic chickens and conventional chickens are raised in different environments. Usually conventional chicken are raised indoors which are more isolated compared to outdoors where organic chicken are raised. The second reason is that antibiotics could not be used in organic chicken feed, but 32 different antibiotics can be used on conventional chicken without veterinary prescription (1). Third reason is that organic chicken are usually slaughtered in small slaughterhouses because of the small amount of products sold each day in the

market. A USDA study reported that *Salmonella* positive rate was related with the size of slaughterhouses, and small slaughterhouses (fewer than 200 birds slaughtered per day) had a much higher *Salmonella* contamination compared with larger slaughterhouses due to possible poor hygienic conditions (16).

Our findings also showed that it is necessary to pick and test at least three *Salmonella* colonies from each positive sample since 16% (24/148) chicken samples contained different *Salmonella* serotypes and 19% (28/148) chicken samples contained *Salmonella* isolates with different PFGE patterns.

Serotyping, PFGE and antibiotics susceptibility testing showed that organic and conventional *Salmonella* isolates might have different origins. *S. Kentucky*, *S. Heidelberg* and *S. Typhimurium* were the most frequently isolated serotypes from organic chicken samples, whereas, in the conventional chicken samples, *S. Typhimurium* and *S. Kentucky* were the two predominant serotypes, and *S. Heidelberg* was only recovered from one sample. Only 5 common PFGE patterns were identified from both organic and conventional chicken samples. Even among isolates from the same serotypes, such as *S. Kentucky* and *S. Typhimurium*, different PFGE patterns were identified from conventional and organic chicken samples. This was particularly true for *S. Typhimurium* isolates, among which no identical PFGE patterns were shared from organic and conventional chicken samples. Antibiotics susceptibility showed that the conventional chicken had a higher prevalence of *Salmonella* resistant to antibiotics than the organic chicken samples. For example, all 12 *S. Typhimurium*- positive conventional chicken samples contained *Typhimurium* isolates resistant to at least 5 different antibiotics, but no *Typhimurium* isolates resistant to more than 4 antibiotics were

recovered from 19 Typhimurium-positive organic chicken samples. These differences are related with the antibiotic uses during conventional chicken growth.

Over half of the antibiotics produced in the United States are used for agricultural purposes (18). A total of 32 antimicrobial compounds, such as penicillin ceftiofur chlortetracycline, are approved for use in conventional broiler feeds in the United States without a veterinary prescription (1). The Animal Health Institute (AHI) estimates that the total animal antimicrobial use in 1999 was 20.42 million pounds that 17.62 million pounds (86%) were used to prevent and treat diseases and 2.8 million pounds (14%) were used for growth promotion (8). It has been shown that antibiotic use in animal husbandry is a driving force for the development of antibiotic resistance(28). The volume of drug uses are closely related with the frequency of antibiotic-resistant mutants(6).

Our results indicate that organic chicken samples were frequently contaminated with *Salmonella*, and that *Salmonella* from organic chicken were more susceptible to antimicrobials commonly used in human and veterinary medicine.

REFERENCES

1. Miller Publishing Company. 2001. Feed Additive Compend. 40: 125-436.
2. National Organic Program. USDA. [Online.] <http://www.ams.usda.gov/nop/NOP/standards.html>. Accessed April 14th, 2004.
3. Standard molecular subtyping of foodborne bacterial pathogens by pulsed-field gel electrophoresis: CDC training manual. Atlanta: Center for Disease Control and prevention, 1998.
4. U.S. Department of Agriculture. Title 15, Subtitle 22. Organically Produced Commodities, Chapter 4. Production Standards for Organic Animal Product.

5. USDA/FSIS Microbiology Laboratory Guidebook, 3rd edition, 1998.
6. **Austin, D. J., K. G. Kristinsson, and R. M. Anderson.** 1999. The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. *Proc Natl Acad Sci U S A* **96**:1152-6.
7. **Bannerman, T. L., G. A. Hancock, F. C. Tenover, and J. M. Miller.** 1995. Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. *J Clin Microbiol* **33**:551-5.
8. **Carnevale, R.** 2001. Antibiotic resistance and food producing animals-Views of the industry. [Online.] Accessed : July 2002.
9. **Corkill, J. E., R. Graham, C. A. Hart, and S. Stubbs.** 2000. Pulsed-field gel electrophoresis of degradation-sensitive DNAs from *Clostridium difficile* PCR ribotype 1 strains. *J Clin Microbiol* **38**:2791-2.
10. **Dickins, M. A., S. Franklin, R. Stefanova, G. E. Schutze, K. D. Eisenach, I. Wesley, and M. D. Cave.** 2002. Diversity of *Campylobacter* isolates from retail poultry carcasses and from humans as demonstrated by pulsed-field gel electrophoresis. *J Food Prot* **65**:957-62.
11. **Donabedian, S., L. A. Thal, P. Bozigar, T. Zervos, E. Hershberger, and M. Zervos.** 2003. Antimicrobial resistance in swine and chickens fed virginiamycin for growth promotion. *J Microbiol Methods* **55**:739-43.
12. **Emborg, H. D., J. S. Andersen, A. M. Seyfarth, S. R. Andersen, J. Boel, and H. C. Wegener.** 2003. Relations between the occurrence of resistance to antimicrobial growth promoters among *Enterococcus faecium* isolated from broilers and broiler meat. *Int J Food Microbiol* **84**:273-84.
13. **Engberg, J., F. M. Aarestrup, D. E. Taylor, P. Gerner-Smidt, and I. Nachamkin.** 2001. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance mechanisms and trends in human isolates. *Emerg Infect Dis* **7**:24-34.
14. **Evans, M. C., and H. C. Wegener.** 2003. Antimicrobial growth promoters and *Salmonella* spp., *Campylobacter* spp. in poultry and swine, Denmark. *Emerg Infect Dis* **9**:489-92.
15. **Friedman, C., S. Reddy, M. Samual, R. Marcus, J. Bender, S. Desai, B. Shiferaw, D. Helfrick, M. Carter, B. Anderson, and M. Hoekstra.** 2000. Presented at the International Conference on Emerging Infectious Diseases, Atlanta, GA, July 16-19, 2000.

16. **Fuzihara, T. O., S. A. Fernandes, and B. D. Franco.** 2000. Prevalence and dissemination of Salmonella serotypes along the slaughtering process in Brazilian small poultry slaughterhouses. *J Food Prot* **63**:1749-53.
17. **Liesegang, A., and H. Tschape.** 2002. Modified pulsed-field gel electrophoresis method for DNA degradation-sensitive Salmonella enterica and Escherichia coli strains. *Int J Med Microbiol* **291**:645-8.
18. **Lipsitch, M., R. S. Singer, and B. R. Levin.** 2002. Antibiotics in agriculture: when is it time to close the barn door? *Proc Natl Acad Sci U S A* **99**:5752-4.
19. **Malorny, B., J. Hoorfar, C. Bunge, and R. Helmuth.** 2003. Multicenter Validation of the Analytical Accuracy of Salmonella PCR: towards an International Standard. *Appl Environ Microbiol* **69**:290-6.
20. **Marano, N. N., S. Rossiter, K. Stamey, K. Joyce, T. J. Barrett, L. K. Tollefson, and F. J. Angulo.** 2000. The National Antimicrobial Resistance Monitoring System (NARMS) for enteric bacteria, 1996-1999: surveillance for action. *J Am Vet Med Assoc* **217**:1829-30.
21. **McAvoy, S.** 2000. Glickman announces national standards for organic food. USDA. [Online.] <http://www.usda.gov/news/releases/2000/12/0425.htm>. Accessed: April 14, 2004.
22. **National Committee for Clinical Laboratory Standards.** 2001. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals - approved standards M31-A. NCCLS, Wayne, PA.
23. **Rahn, K., S. A. De Grandis, R. C. Clarke, S. A. McEwen, J. E. Galan, C. Ginocchio, R. Curtiss, 3rd, and C. L. Gyles.** 1992. Amplification of an invA gene sequence of Salmonella typhimurium by polymerase chain reaction as a specific method of detection of Salmonella. *Mol Cell Probes* **6**:271-9.
24. **Romling, U., and B. Tummeler.** 2000. Achieving 100% typeability of Pseudomonas aeruginosa by pulsed-field gel electrophoresis. *J Clin Microbiol* **38**:464-5.
25. **Silbert, S., L. Boyken, R. J. Hollis, and M. A. Pfaller.** 2003. Improving typeability of multiple bacterial species using pulsed-field gel electrophoresis and thiourea. *Diagn Microbiol Infect Dis* **47**:619-21.
26. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**:2233-9.

27. **Wegener, H. C.** 2003. Antibiotics in animal feed and their role in resistance development. *Curr Opin Microbiol* **6**:439-45.
28. **Witte, W.** 1998. Medical consequences of antibiotic use in agriculture. *Science* **279**:996-7.

Chapter 6 Summary

Each year, pathogens cause an estimate of 76 million cases of foodborne illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States. *Salmonella* and *Escherichia coli* O157 are among most important foodborne pathogens. More than 95% of the cases caused by *Salmonella* and *E. coli* O157:H7 are infected through consumption of contaminated raw or undercooked food products. My Ph.D. research is focused on detection and characterization of *E. coli* O157:H7 and *Salmonella* in food products. We identified cell density-dependent survival phenotype of *E. coli* O157:H7 under acidic conditions, developed food sample preparation procedures for PCR detection of *E. coli* O157:H7, improved the methodology in isolation and identification of *Salmonella* from meat products, characterized antimicrobial resistance of *Salmonella* from retail chickens raised under conventional and organic productions.

In chapter 2, the cell density-dependent acid sensitivity phenotypes of *E. coli* strains K-12 and O157:H7 were examined during stationary growth phase. High cell density suspensions (10^9 CFU/ml) survived acid-challenge very poorly ($\leq 0.004\%$ survival) in Luria-Bertani broth (pH 2.5, 37 °C, 2 h). The same cultures, when subjected to an identical acid-challenge at lower cell density (10^6 CFU/ml), showed higher survival ($\geq 15\%$). We analyzed the cell density-dependent acid sensitive phenotype of *E. coli*, with reference to three possible mechanisms of acid resistance. There was no evidence of any diffusible substance released from dead cells, which could influence the cell density-dependent acid-survival phenotype. Nor was there evidence for the involvement of the alternative sigma-transcription factor (*rpoS*). Instead, cell-density dependent acid-

survival phenotype was associated with induction of glutamate- and arginine-decarboxylase acid-survival pathways and concomitant availability of glutamate and arginine during acid-challenge. The data revealed that during acid-challenge at high cell density, the limited availability of glutamate and/or arginine creates an illusion of cell density-dependent acid sensitive phenotype.

In chapter 3, an improved, rapid and sensitive sample preparation method for PCR-based detection of *E. coli* O157:H7 in ground beef was developed, fresh ground beef samples were experimentally inoculated with varying concentrations of *E. coli* O157:H7. PCR inhibitors were removed and bacterial cells were concentrated by filtration, centrifugation, and lysed using enzymatic digestion and successive freeze/thaw cycles. DNA was purified and concentrated via phenol/chloroform extraction and the shiga toxin 1 gene (*stx1*) was amplified using PCR to evaluate the sample preparation method. Without prior enrichment of cells in broth media, the detection limit was 10^3 CFU/g beef. When a 6-h enrichment step was incorporated, the detection limit was 1 CFU/g beef. Total time required from beginning to end of the procedure is 12 h. The sample preparation method developed enabled substantially improved sensitivity in the PCR-based detection of *E. coli* O157:H7 in ground beef, as compared to previous reports.

In chapter 4, an improved procedure based on the method described in the USDA/Food Safety Inspection Service (FSIS) Microbiological Laboratory Guidebook was developed to isolate *Salmonella* from poultry products. The modified method was evaluated using the standard procedures of the FDA/Bacteriological Analytical Manual (BAM) and USDA/FSIS Microbiological Laboratory Guidebook. Greater than 95% of the presumptive *Salmonella* colonies isolated using the modified method from chicken

samples were confirmed as *Salmonella* by PCR/ API 20E tests. Its high specificity significantly reduced the time for isolation and identification of *Salmonella* from poultry products (48 h compared to 3-5 days); thus, the costs were reduced. Since the method has a high specificity, *Salmonella* isolates may be further subtyped by serotyping and/or pulsed-field gel electrophoresis (PFGE) without biochemical confirmation. Using this method, we were able to recover *Salmonella* from 29 of 44 (66%) retail organic chicken samples.

In chapter 5, *Salmonella* in retail organic chicken samples were isolated and characterized by serotyping, antimicrobial-susceptibility testing, and pulsed-field gel electrophoresis (PFGE). Among 198 organic chicken samples, 121 (61%) were *Salmonella* positive, compared to 27 (44%) among 61 conventional chicken samples. Kentucky, Heideburg, and Typhimurium were top serotypes identified and accounted for more than 60% of all *Salmonella* isolates from chicken samples. There were 37 PFGE patterns identified among isolates from 148 chicken samples, of which 14 patterns were found only in conventional chicken samples, 18 patterns were found only in organic chicken samples, and 5 patterns were shared by both conventional and organic chicken samples. Antimicrobial-susceptibility testing results showed that antimicrobial resistance in *Salmonella* was associated with serotypes. All *S. Typhimurium* positive conventional chicken samples contained isolates that were resistant to at least one antimicrobial compared to only 21% of organic chicken samples. The similar trend was found among other serotypes. Our results indicate that organic chicken samples were frequently contaminated with *Salmonella*, and that *Salmonella* from organic chicken were more susceptible to antimicrobials commonly used in human and veterinary medicine.

MASTER REFERENCES

References in chapter 1

1. FDA Bacteriological analytical manual. [Online.] <http://www.foodinfonet.com/publication/fdaBAM.htm>. Accessed April 12, 2004.
2. NARMS 1999 Annual Report. CDC. [Online.] <http://www.cdc.gov/ncidod/dbmd/narms/annual/1999/html/99sum.htm>. Accessed April 12, 2004.
3. Preliminary FoodNet data on the incidence of foodborne illnesses--selected sites, United States, 2000. *MMWR Morb Mortal Wkly Rep* **50**:241-6.
4. Recall Notification Report. Food Safety and Inspection Service, United States Department of Agriculture, Washington, DC. [Online.] <http://www.fsis.usda.gov/OA/recalls/rnrfiles/rnr055-2002.htm>. Accessed April 12, 2004.
5. USDA/FSIS Microbiology laboratory guidebook. [Online.] <http://www.fsis.usda.gov/OPHS/microlab/mlgbook.htm>. Accessed April 12, 2004.
6. **Bearson, S., B. Bearson, and J. W. Foster.** 1997. Acid stress responses in enterobacteria. *FEMS Microbiol Lett* **147**:173-80.
7. **Benjamin, M. M., and A. R. Datta.** 1995. Acid tolerance of enterohemorrhagic *Escherichia coli*. *Appl Environ Microbiol* **61**:1669-72.
8. **Besser, R. E., S. M. Lett, J. T. Weber, M. P. Doyle, T. J. Barrett, J. G. Wells, and P. M. Griffin.** 1993. An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. *Jama* **269**:2217-20.
9. **Bolton, D. J., T. Catarama, C. Byrne, J. J. Sheridan, D. A. McDowell, and I. S. Blair.** 2002. The ineffectiveness of organic acids, freezing and pulsed electric fields to control *Escherichia coli* O157:H7 in beef burgers. *Lett Appl Microbiol* **34**:139-43.
10. **Brunder, W., H. Schmidt, and H. Karch.** 1996. KatP, a novel catalase-peroxidase encoded by the large plasmid of enterohaemorrhagic *Escherichia coli* O157:H7. *Microbiol* **142** (Pt 11):3305-15.
11. **Calicioglu, M., N. G. Faith, D. R. Buege, and J. B. Luchansky.** 2002. Viability of *Escherichia coli* O157:H7 during manufacturing and storage of a fermented, semidry soudjouk-style sausage. *J Food Prot* **65**:1541-4.

12. **Carnevale, R.** 2001. Antibiotic resistance and food producing animals-Views of the industry. [Online.] <http://www.ahi.org>. Accessed July 1st, 2002.
13. **Castanie-Cornet, M. P., T. A. Penfound, D. Smith, J. F. Elliott, and J. W. Foster.** 1999. Control of acid resistance in *Escherichia coli*. *J Bacteriol* **181**:3525-35.
14. **CDC.** 2003. *Salmonella* Surveillance Summary, 2002. US Department of Health and Human Services.
15. **Datta, A. R., and M. M. Benjamin.** 1997. Factors controlling acid tolerance of *Listeria monocytogenes*: effects of nisin and other ionophores. *Appl Environ Microbiol* **63**:4123-6.
16. **I. Dmitri, A. Plamen, W. Ebtisam, S. Stephen.** 2000. Application of electrochemical biosensors for detection of food pathogenic bacteria. *Electroanalysis* **12**:317-325.
17. **Donnenberg, M. S., and T. S. Whittam.** 2001. Pathogenesis and evolution of virulence in enteropathogenic and enterohemorrhagic *Escherichia coli*. *J Clin Invest* **107**:539-48.
18. **Edwards, P. R., and W. H. Ewing.** 1972. Identification of *Enterobacteriaceae*. Burgess Publishing Co., Minneapolis, Minn.
19. **Eiguer, T., and M. I. Caffer.** 1988. [*Salmonella* taxonomy]. *Rev Argent Microbiol* **20**:151-4.
20. **Elder, R. O., J. E. Keen, G. R. Siragusa, G. A. Barkocy-Gallagher, M. Koohmaraie, and W. W. Laegreid.** 2000. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proceedings of the National Academy of Sciences of the United States of America* **97**:2999-3003.
21. **Foster, J. W.** 1995. Low pH adaptation and the acid tolerance response of *Salmonella* Typhimurium. *Crit Rev Microbiol* **21**:215-37.
22. **Foster, J. W.** 1999. When protons attack: microbial strategies of acid adaptation. *Curr Opin Microbiol* **2**:170-4.
23. **Glass, K. A., J. M. Loeffelholz, J. P. Ford, and M. P. Doyle.** 1992. Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. *Appl Environ Microbiol* **58**:2513-6.
24. **Gooding, C. M., and P. V. Choudary.** 1997. Rapid and sensitive immunomagnetic separation-polymerase chain reaction method for the detection of *Escherichia coli* O157:H7 in raw milk and ice-cream. *J Dairy Res* **64**:87-93.

25. **Gorden, J., and P. L. Small.** 1993. Acid resistance in enteric bacteria. *Infect Immun* **61**:364-7.
26. **Griffin, P. M., S. M. Ostroff, R. V. Tauxe, K. D. Greene, J. G. Wells, J. H. Lewis, and P. A. Blake.** 1988. Illnesses associated with *Escherichia coli* O157:H7 infections. A broad clinical spectrum. *Ann Intern Med* **109**:705-12.
27. **Griffin, P. M., and R. V. Tauxe.** 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol Rev* **13**:60-98.
28. **Hathcox, A. K., L. R. Beuchat, and M. P. Doyle.** 1995. Death of enterohemorrhagic *Escherichia coli* O157:H7 in real mayonnaise and reduced-calorie mayonnaise dressing as influenced by initial population and storage temperature. *Appl Environ Microbiol* **61**:4172-7.
29. **Helms, M., P. Vastrup, P. Gerner-Smidt, and K. Molbak.** 2002. Excess mortality associated with antimicrobial drug-resistant *Salmonella* Typhimurium. *Emerg Infect Dis* **8**:490-5.
30. **Jay, J. M.** 2000. *Modern Food Microbiology*, sixth edition. Aspen Publishers, Inc, Gaithersburg, MD.
31. **Jordan, K. N., L. Oxford, and C. P. O'Byrne.** 1999. Survival of low-pH stress by *Escherichia coli* O157:H7: correlation between alterations in the cell envelope and increased acid tolerance. *Appl Environ Microbiol* **65**:3048-55.
32. **Kim, J., J. Nietfeldt, J. Ju, J. Wise, N. Fegan, P. Desmarchelier, and A. K. Benson.** 2001. Ancestral divergence, genome diversification, and phylogeographic variation in subpopulations of sorbitol-negative, beta-glucuronidase-negative enterohemorrhagic *Escherichia coli* O157. *J Bacteriol* **183**:6885-97.
33. **Le Minor, L., M. Y. Popoff, B. Laurent, and D. Hermant.** 1986. [Characterization of a 7th subspecies of *Salmonella*: *S. Choleraesuis* subsp. indica subsp. nov.]. *Ann Inst Pasteur Microbiol* **137B**:211-7.
34. **Le Minor, L., M. Veron, and M. Popoff.** 1982. [The taxonomy of *Salmonella*]. *Ann Microbiol (Paris)* **133**:223-43.
35. **Liebana, E., L. Garcia-Migura, C. Clouting, F. A. Clifton-Hadley, E. Lindsay, E. J. Threlfall, S. W. McDowell, and R. H. Davies.** 2002. Multiple genetic typing of *Salmonella enterica* serotype typhimurium isolates of different phage types (DT104, U302, DT204b, and DT49) from animals and humans in England, Wales, and Northern Ireland. *J Clin Microbiol* **40**:4450-6.

36. **Lin, J., I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster.** 1995. Comparative analysis of extreme acid survival in *Salmonella* Typhimurium, *Shigella flexneri*, and *Escherichia coli*. *J Bacteriol* **177**:4097-104.
37. **Lin, J., M. P. Smith, K. C. Chapin, H. S. Baik, G. N. Bennett, and J. W. Foster.** 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. *Appl Environ Microbiol* **62**:3094-100.
38. **Marques, P. A., D. Worcman-Barninka, S. C. Lannes, and M. Landgraf.** 2001. Acid tolerance and survival of *Escherichia coli* O157:H7 inoculated in fruit pulps stored under refrigeration. *J Food Prot* **64**:1674-8.
39. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. *Emerg Infect Dis* **5**:607-25.
40. **Meng, J., M. P. Doyle, T. Zhao, and S. Zhao.** 2001. Enterohemorrhagic *Escherichia coli*. In *Food microbiology: fundamentals and frontiers*. American Society of Microbiology Press, Washington, DC.
41. **O'Hara, G. W., and A. R. Glenn.** 1994. The adaptive acid tolerance response in root nodule bacteria and *Escherichia coli*. *Arch Microbiol* **161**:286-92.
42. **O'Ryan, M.** 1996. An outbreak of *Salmonella* infection from ice cream. *N Engl J Med* **335**:824; author reply 824-5.
43. **Panico, M. G., F. Primiano, F. Nappi, and F. Attena.** 1999. An outbreak of *Salmonella* Enteritidis food poisoning from a commercially produced cheese. *Euro Surveill* **4**:47-48.
44. **Parham, N., J. Spencer, D. Taylor, H. Ternent, G. Innocent, D. Mellor, M. Roberts, and A. Williams.** 2003. An adapted ImmunoMagnetic cell separation method for use in quantification of *Escherichia coli* O157:H7 from bovine faeces. *J Microbiol Methods* **53**:1-9.
45. **Raja, N., M. Goodson, W. C. Chui, D. G. Smith, and R. J. Rowbury.** 1991. Habituation to acid in *Escherichia coli*: conditions for habituation and its effects on plasmid transfer. *J Appl Bacteriol* **70**:59-65.
46. **Rajashekara, G., E. Haverly, D. A. Halvorson, K. E. Ferris, D. C. Lauer, and K. V. Nagaraja.** 2000. Multidrug-resistant *Salmonella* Typhimurium DT104 in poultry. *J Food Prot* **63**:155-61.
47. **Richard, H. T., and J. W. Foster.** 2003. Acid resistance in *Escherichia coli*. *Adv Appl Microbiol* **52**:167-86.

48. **Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. McGee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen.** 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *New England Journal of Medicine* **308**:681-685.
49. **Ryu, J. H., and L. R. Beuchat.** 1998. Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. *Int J Food Microbiol* **45**:185-93.
50. **Sagoo, S. K., C. L. Little, L. Ward, I. A. Gillespie, and R. T. Mitchell.** 2003. Microbiological study of ready-to-eat salad vegetables from retail establishments uncovers a national outbreak of salmonellosis. *J Food Prot* **66**:403-9.
51. **Salyers, A. A.** 2002. An overview of the genetic basis of antibiotic resistance in bacteria and its implications for agriculture. *Anim Biotechnol* **13**:1-5.
52. **Sarna, M., G. Dowse, G. Evans, and C. Guest.** 2002. An outbreak of *Salmonella* Typhimurium PT135 gastroenteritis associated with a minimally cooked dessert containing raw eggs. *Commun Dis Intell* **26**:32-7.
53. **Schmidt, H., and M. Hensel.** 2004. Pathogenicity islands in bacterial pathogenesis. *Clin Microbiol Rev* **17**:14-56.
54. **Schmidt, H., C. Kernbach, and H. Karch.** 1996. Analysis of the EHEC hly operon and its location in the physical map of the large plasmid of enterohaemorrhagic *Escherichia coli* O157:h7. *Microbiology* **142 (Pt 4)**:907-14.
55. **Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden.** 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella* Typhimurium. *Proc Natl Acad Sci U S A* **93**:2593-7.
56. **Tauxe, R. V.** 2002. Emerging foodborne pathogens. *Int J Food Microbiol* **78**:31-41.
57. **Tomoyasu, T.** 1998. Improvement of the immunomagnetic separation method selective for *Escherichia coli* O157 strains. *Appl Environ Microbiol* **64**:376-82.
58. **Whittam, T. S., M. L. Wolfe, I. K. Wachsmuth, F. Orskov, I. Orskov, and R. A. Wilson.** 1993. Clonal relationships among *Escherichia coli* strains that cause hemorrhagic colitis and infantile diarrhea. *Infect. Immun.* **61**:1619-1629.
59. **Witte, W.** 1998. Medical consequences of antibiotic use in agriculture. *Science* **279**:996-7.
60. **Zhao, T., M. P. Doyle, P. J. Fedorka-Cray, P. Zhao, and S. Ladely.** 2002. Occurrence of *Salmonella enterica* serotype typhimurium DT104A in retail ground beef. *J Food Prot* **65**:403-7.

61. **Zueva, N. N., T. A. Popova, A. Sypchenko, and L. I. Shishkina.** 2000. [The epidemiology of acute intestinal infections complicated by the development of the hemolytic-uremic syndrome in Tula Province]. *Zh Mikrobiol Epidemiol Immunobiol*:105-7.

References in chapter 2

1. **Abdul-Raouf, U. M., L. R. Beuchat, and M. S. Ammar.** 1993. Survival and growth of *Escherichia coli* O157:H7 in ground, roasted beef as affected by pH, acidulants, and temperature. *Appl Environ Microbiol.* **59**:2364-8.
2. **Arnold, K. W., and C. W. Kaspar.** 1995. Starvation- and stationary-phase-induced acid tolerance in *Escherichia coli* O157:H7. *Appl Environ Microbiol.* **61**:2037-9.
3. **Bang, I. S., B. H. Kim, J. W. Foster, and Y. K. Park.** 2000. OmpR regulates the stationary-phase acid tolerance response of *Salmonella enterica* serovar typhimurium. *J Bacteriol.* **182**:2245-52.
4. **Bearson, S., B. Bearson, and J. W. Foster.** 1997. Acid stress responses in enterobacteria. *FEMS Microbiol Lett.* **147**:173-80.
5. **Beumer, R. R., J. de Vries, and F. M. Rombouts.** 1992. *Campylobacter jejuni* non-culturable coccoid cells. *Int J Food Microbiol.* **15**:153-63.
6. **Castanie-Cornet, M. P., T. A. Penfound, D. Smith, J. F. Elliott, and J. W. Foster.** 1999. Control of acid resistance in *Escherichia coli*. *J Bacteriol.* **181**:3525-35.
7. **Datta, A. R., and M. M. Benjamin.** 1999. Cell density dependent acid sensitivity in stationary phase cultures of enterohemorrhagic *Escherichia coli* O157:H7. *FEMS Microbiol Lett.* **181**:289-95.
8. **De Kievit, T. R., and B. H. Iglewski.** 2000. Bacterial quorum sensing in pathogenic relationships. *Infect Immun.* **68**:4839-49.
9. **Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J. Harwood, and D. G. Guiney.** 1992. The alternative sigma factor katF (rpoS) regulates *Salmonella* virulence. *Proc Natl Acad Sci U S A.* **89**:11978-82.
10. **Foster, J. W.** 1999. When protons attack: microbial strategies of acid adaptation. *Curr Opin Microbiol.* **2**:170-4.

11. **Foster, J. W., and H. K. Hall.** 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella* Typhimurium. *J Bacteriol.* **173**:5129-35.
12. **Foster, J. W., and M. Moreno.** 1999. Inducible acid tolerance mechanisms in enteric bacteria. *Novartis Found Symp.* **221**:55-69.
13. **Glass, K. A., J. M. Loeffelholz, J. P. Ford, and M. P. Doyle.** 1992. Fate of *Escherichia coli* O157:H7 as affected by pH or sodium chloride and in fermented, dry sausage. *Appl Environ Microbiol.* **58**:2513-6.
14. **Gorden, J., and P. L. Small.** 1993. Acid resistance in enteric bacteria. *Infect Immun.* **61**:364-7.
15. **Hersh, B. M., F. T. Farooq, D. N. Barstad, D. L. Blankenhorn, and J. L. Slonczewski.** 1996. A glutamate-dependent acid resistance gene in *Escherichia coli*. *J Bacteriol.* **178**:3978-81.
16. **Hussain, N. H., M. Goodson, and R. J. Rowbury.** 1998. Recent advances in biology: intercellular communication and quorum sensing in micro-organisms. *Sci Prog.* **81**:69-80.
17. **Lin, J., I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster.** 1995. Comparative analysis of extreme acid survival in *Salmonella* Typhimurium, *Shigella flexneri*, and *Escherichia coli*. *J Bacteriol.* **177**:4097-104.
18. **O'Hara, G. W., and A. R. Glenn.** 1994. The adaptive acid tolerance response in root nodule bacteria and *Escherichia coli*. *Arch Microbiol.* **161**:286-92.
19. **Price, S. B., C. M. Cheng, C. W. Kaspar, J. C. Wright, F. J. DeGraves, T. A. Penfound, M. P. Castanie-Cornet, and J. W. Foster.** 2000. Role of rpoS in acid resistance and fecal shedding of *Escherichia coli* O157:H7. *Appl Environ Microbiol.* **66**:632-7.
20. **Raja, N., M. Goodson, W. C. Chui, D. G. Smith, and R. J. Rowbury.** 1991. Habituation to acid in *Escherichia coli*: conditions for habituation and its effects on plasmid transfer. *J Appl Bacteriol.* **70**:59-65.
21. **Rao, N. N., S. Liu, and A. Kornberg.** 1998. Inorganic Polyphosphate in *Escherichia coli*: the Phosphate Regulon and the Stringent Response. *J. Bacteriol.* **180**:2186-2193.
22. **Rowbury, R. J.** 1999. Acid tolerance induced by metabolites and secreted proteins, and how tolerance can be counteracted. *Novartis Found Symp.* **221**:93-106.

23. **Rowbury, R. J.** 2000. Killed cultures of *Escherichia coli* can protect living organisms from acid stress. *Microbiol.* **146**:1759-60.
24. **Small, P., D. Blankenhorn, D. Welty, E. Zinser, and J. L. Slonczewski.** 1994. Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of *rpoS* and growth pH. *J. Bacteriol.* **176**:1729-1737.
25. **Sperandio, V., J. L. Mellies, W. Nguyen, S. Shin, and J. B. Kaper.** 1999. Quorum sensing controls expression of the type III secretion gene transcription and protein secretion in enterohemorrhagic and enteropathogenic *Escherichia coli*. *Proc Natl Acad Sci U S A.* **96**:15196-201.
26. **Surette, M. G., and B. L. Bassler.** 1999. Regulation of autoinducer production in *Salmonella* Typhimurium. *Mol Microbiol.* **31**:585-95.
27. **Surette, M. G., M. B. Miller, and B. L. Bassler.** 1999. Quorum sensing in *Escherichia coli*, *Salmonella* Typhimurium, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production. *Proc Natl Acad Sci U S A.* **96**:1639-44.

References in chapter 3

1. USDA/FSIS Microbiology Laboratory Guidebook, 1998. 3rd Edition, 5.1-5.10.
2. **Abolmaaty, A., El-Shemy, M.G., Khallaf, M.F. and Levin, R.E.** 1998. Effect of lysing methods and their variables on the yield of *Escherichia coli* O157:H7 DNA and its PCR amplification. *J. Microbiol. Methods* 34:133-141.
3. **Abu Al-Soud, W., and P. Radstrom.** 2000. Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. *J Clin Microbiol* 38:4463-70.
4. United States Department of Agriculture. Recall Notification Report. <http://www.fsis.usda.gov/OA/recalls/rnrfiles/rnr055-2002.htm> 055-2002. 2002. Food Safety and Inspection Service, United States Department of Agriculture, Washington, DC. Accessed April 12, 2004.
5. **Barns, S. M., R. E. Fundyga, M. W. Jeffries, and N. R. Pace.** 1994. Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment. *Proc Natl Acad Sci U S A* 91:1609-13.
6. **Bhaduri, S. a. C., B.** 2001. Sample preparation methods for PCR detection of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* on beef chuck shoulder using a single enrichment medium. *Mol. Cell Probes* 15:267-274.

7. **Clark, J. A.** 1980. The influence of increasing numbers of non-indicator organisms upon the detection of indicator organisms by the membrane filter and presence-absence tests. *Can. J. Microbiol.* 26:827-832.
8. **Elder, R. O., J. E. Keen, G. R. Siragusa, G. A. Barkocy-Gallagher, M. Koohmaraie, and W. W. Laegreid.** 2000. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *Proc Natl Acad Sci U S A* 97:2999-3003.
9. **Feng, P.** 2001. Development and impact of rapid methods for detection of foodborne pathogens. In *Food microbiology: fundamentals and frontiers*. ASM Press, Washington, DC.
10. **Ge, B., C. Larkin, S. Ahn, M. Jolley, M. Nasir, J. Meng, and R. H. Hall.** 2002. Identification of *Escherichia coli* O157:H7 and other enterohemorrhagic serotypes by EHEC- hly A targeting, strand displacement amplification, and fluorescence polarization. *Mol Cell Probes* 16:85-92.
11. **Griffin, P. M.** 1995. *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli*. In *Infections of the gastrointestinal tract*. Raven Press, Ltd, New York.
12. **Kaclikova, E., Kuchta, T.V., Kay, H. and Gray, D.** 2001. Separation of *Listeria* from cheese and enrichment media using antibody-coated microbeads and centrifugation. *J. Microbiol. Methods* 46:63-67.
13. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. *Emerg Infect Dis* 5:607-25.
14. **Meng, J., Doyle, M. P., Zhao, T. and Zhao, S.** 2001. Enterohemorrhagic *Escherichia coli*. In *Food microbiology: fundamentals and frontiers*. ASM Press, Washington, DC.
15. **O'Brien, A. D., V. L. Tesh, A. Donohue-Rolfe, M. P. Jackson, S. Olsnes, K. Sandvig, A. A. Lindberg, and G. T. Keusch.** 1992. Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. *Curr Top Microbiol Immunol* 180:65-94.
16. **Pass, M. A., R. Odedra, and R. M. Batt.** 2000. Multiplex PCRs for identification of *Escherichia coli* virulence genes. *J Clin Microbiol* 38:2001-4.
17. **Riley, L. W., Remis, R.S., Helgerson, S.D., McGee, H.B., Wells, J.G., Davis, B.R., Hebert, R.J., Olcott, E.S., Johnson, L.M., Hargrett, N.T., Blake, P.A.**

- and Cohen, M.L.** 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. N. Engl. J. Med 308:681-685.
18. **Seo, K. H., R. E. Brackett, J. F. Frank, and S. Hilliard.** 1998. Immunomagnetic separation and flow cytometry for rapid detection of *Escherichia coli* O157:H7. J Food Prot 61:812-6.
 19. **Simon M.C., G. D. I., and Cook N.** 1996. DNA extraction and PCR methods for the detection of *Listeria monocytogenes* in cold-smoked salmon. Appl. Environ. Microbiol. 62:822-824.
 20. **Tomoyasu, T.** 1998. Improvement of the immunomagnetic separation method selective for *Escherichia coli* O157 strains. Appl. Environ. Microbiol 64:376-382.
 21. **Tsai, Y. L. a. O., B.H.** 1991. Rapid method for direct extraction of DNA from soil and sediments. Appl. Environ. Microbiol 57:1070-1074.
 22. **Witham, P. K., Yamashiro, C.T., Livak, K.J. and Batt, C.A.** 1996. A PCR-based assay for the detection of *Escherichia coli* Shiga-like toxin genes in ground beef. Appl. Environ. Microbiol. 62:1347-1353.

References in chapter 4

1. Microbiology - General guidance on methods for the detection of *Salmonella* spp., 3rd edition. International Organization for Standardization, Geneva, Switzerland. 679, 1993.
2. **Antunes, P., C. Reu, J. C. Sousa, L. Peixe, and N. Pestana.** 2003. Incidence of *Salmonella* from poultry products and their susceptibility to antimicrobial agents. Int J Food Microbiol **82**:97-103.
3. **Blivet, D., G. Salvat, F. Humbert, and P. Colin.** 1997. Evaluation of a new enrichment broth for the isolation of *Salmonella* spp. from poultry products. Int J Food Microbiol **38**:211-6.
4. **Coker, C., O. O. Bakare, and H. L. Mobley.** 2000. H-NS is a repressor of the *Proteus mirabilis* urease transcriptional activator gene ureR. J Bacteriol **182**:2649-53.
5. **Dattelbaum, J. D., C. V. Lockatell, D. E. Johnson, and H. L. Mobley.** 2003. UreR, the transcriptional activator of the *Proteus mirabilis* urease gene cluster, is required for urease activity and virulence in experimental urinary tract infections. Infect Immun **71**:1026-30.

6. **Fung, D. Y., and A. A. Kraft.** 1970. A rapid and simple method for the detection and isolation of *Salmonella* from mixed cultures and poultry products. *Poult Sci* **49**:46-54.
7. **Funk, J. A., P. R. Davies, and M. A. Nichols.** 2000. The effect of fecal sample weight on detection of *Salmonella enterica* in swine feces. *J Vet Diagn Invest* **12**:412-8.
8. **Fuzihara, T. O., S. A. Fernandes, and B. D. Franco.** 2000. Prevalence and dissemination of *Salmonella* serotypes along the slaughtering process in Brazilian small poultry slaughterhouses. *J Food Prot* **63**:1749-53.
9. **Jorgensen, F., R. Bailey, S. Williams, P. Henderson, D. R. Wareing, F. J. Bolton, J. A. Frost, L. Ward, and T. J. Humphrey.** 2002. Prevalence and numbers of *Salmonella* and *Campylobacter* spp. on raw, whole chickens in relation to sampling methods. *Int J Food Microbiol* **76**:151-64.
10. **Kalina, G. P.** 1983. [Combined media for the simplified and rapid identification of bacteria of the *Proteus-Providencia* group]. *Zh Mikrobiol Epidemiol Immunobiol*:53-7.
11. **Karras, D. J.** 2000. Incidence of foodborne illnesses: preliminary data from the foodborne diseases active surveillance network (FoodNet). *Ann Emerg Med* **35**:92-3.
12. **Knox, R., P. H. Gell, and M. R. Pollack.** 1942. Selective media for organisms of the *Salmonella* group. *J. Pathol. Bacteriol.* **54**:469-483.
13. **Malorny, B., J. Hoorfar, C. Bunge, and R. Helmuth.** 2003. Multicenter Validation of the Analytical Accuracy of *Salmonella* PCR: towards an International Standard. *Appl Environ Microbiol* **69**:290-6.
14. **Munoz, A., E. Frapolli, M. A. Morinigo, J. J. Borrego, and P. Romero.** 1987. Isolation of *Salmonella* using three selective enrichment broths from sausages naturally contaminated. Its relationship with other microorganisms that produce food toxic-infections. *Zentralbl Bakteriol Mikrobiol Hyg [B]* **185**:105-11.
15. **Oltmann, L. F., V. P. Claassen, P. Kastelein, W. N. Reijnders, and A. H. Stouthamer.** 1979. Influence of tungstate on the formation and activities of four reductases in *Proteus mirabilis*: identification of two new molybdo-enzymes: chlorate reductase and tetrathionate reductase. *FEBS Lett* **106**:43-6.
16. **Oltmann, L. F., and A. H. Stouthamer.** 1975. Reduction of tetrathionate, trithionate and thiosulphate, and oxidation of sulphide in *proteus mirabilis*. *Arch Microbiol* **105**:135-42.

17. **Papavassiliou, J., V. Samaraki-Lyberopoulou, and G. Piperakis.** 1969. Production of tetrathionate reductase by *Salmonella*. *Can J Microbiol* **15**:238-40.
18. **Rahn, K., S. A. De Grandis, R. C. Clarke, S. A. McEwen, J. E. Galan, C. Ginocchio, R. Curtiss, 3rd, and C. L. Gyles.** 1992. Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol Cell Probes* **6**:271-9.
19. **Read, S. C., R. J. Irwin, C. Poppe, and J. Harris.** 1994. A comparison of two methods for isolation of *Salmonella* from poultry litter samples. *Poult Sci* **73**:1617-21.
20. **Rose, B. E., W. E. Hill, R. Umholtz, G. M. Ransom, and W. O. James.** 2002. Testing for *Salmonella* in raw meat and poultry products collected at federally inspected establishments in the United States, 1998 through 2000. *J Food Prot* **65**:937-47.
21. **Sauter, E. A., C. F. Petersen, E. E. Steele, J. F. Parkinson, J. E. Dixon, and R. C. Stroh.** 1981. The airborne microflora of poultry houses. *Poult Sci* **60**:569-74.
22. **Sofos, J. N., S. L. Kochevar, J. O. Reagan, and G. C. Smith.** 1999. Incidence of *Salmonella* on beef carcasses relating to the U.S. meat and poultry inspection regulations. *J Food Prot* **62**:467-73.
23. **Tirado, C., and K. Schmidt.** 2001. WHO surveillance programme for control of foodborne infections and intoxications: preliminary results and trends across greater Europe. World Health Organization. *J Infect* **43**:80-4.
24. **Trevejo, R. T., J. G. Courtney, M. Starr, and D. J. Vugia.** 2003. Epidemiology of salmonellosis in California, 1990-1999: morbidity, mortality, and hospitalization costs. *Am J Epidemiol* **157**:48-57.
25. **Uyttendaele, M., P. De Troy, and J. Debevere.** 1999. Incidence of *Salmonella*, *Campylobacter jejuni*, *Campylobacter coli*, and *Listeria monocytogenes* in poultry carcasses and different types of poultry products for sale on the Belgian retail market. *J Food Prot* **62**:735-40.
26. **Vassiliadis, P., C. Mavromati, D. Trichopoulos, V. Kalapothaki, and J. Papadakis.** 1987. Comparison of procedures based upon Rappaport-Vassiliadis medium with those using Muller-Kauffmann medium containing Teepol for the isolation of *Salmonella sp.* *Epidemiol Infect* **99**:143-7.
27. **Veldman, A., H. A. Vahl, G. J. Borggreve, and D. C. Fuller.** 1995. A survey of the incidence of *Salmonella* species and *Enterobacteriaceae* in poultry feeds and feed components. *Vet Rec* **136**:169-72.

28. **Wallace, D. J., T. Van Gilder, S. Shallow, T. Fiorentino, S. D. Segler, K. E. Smith, B. Shiferaw, R. Etzel, W. E. Garthright, and F. J. Angulo.** 2000. Incidence of foodborne illnesses reported by the foodborne diseases active surveillance network (FoodNet)-1997. FoodNet Working Group. *J Food Prot* **63**:807-9.
29. **Waltman, W. D., and E. T. Mallinson.** 1995. Isolation of *Salmonella* from poultry tissue and environmental samples: a nationwide survey. *Avian Dis* **39**:45-54.
30. **Zhao, C., B. Ge, J. De Villena, R. Sudler, E. Yeh, S. Zhao, D. G. White, D. Wagner, and J. Meng.** 2001. Prevalence of *Campylobacter* spp., *Escherichia coli*, and *Salmonella* serovars in retail chicken, turkey, pork, and beef from the Greater Washington, D.C., area. *Appl Environ Microbiol* **67**:5431-6.

References in chapter 5

1. Miller Publishing Company. 2001. *Feed Additive Compend*. 40: 125-436.
2. National Organic Program. USDA. [Online.] <http://www.ams.usda.gov/nop/NOP/standards.html>. Accessed April 14th, 2004.
3. Standard molecular subtyping of foodborne bacterial pathogens by pulsed-field gel electrophoresis: CDC training manual. Atlanta: Center for Disease Control and prevention, 1998.
4. U.S. Department of Agriculture. Title 15, Subtitle 22. Organically Produced Commodities, Chapter 4. Production Standards for Organic Animal Product.
5. USDA/FSIS Microbiology Laboratory Guidebook, 3rd edition, 1998.
6. **Austin, D. J., K. G. Kristinsson, and R. M. Anderson.** 1999. The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. *Proc Natl Acad Sci U S A* **96**:1152-6.
7. **Bannerman, T. L., G. A. Hancock, F. C. Tenover, and J. M. Miller.** 1995. Pulsed-field gel electrophoresis as a replacement for bacteriophage typing of *Staphylococcus aureus*. *J Clin Microbiol* **33**:551-5.
8. **Carnevale, R.** 2001. Antibiotic resistance and food producing animals-Views of the industry. [Online.] Accessed : July 2002.
9. **Corkill, J. E., R. Graham, C. A. Hart, and S. Stubbs.** 2000. Pulsed-field gel electrophoresis of degradation-sensitive DNAs from *Clostridium difficile* PCR ribotype 1 strains. *J Clin Microbiol* **38**:2791-2.

10. **Dickins, M. A., S. Franklin, R. Stefanova, G. E. Schutze, K. D. Eisenach, I. Wesley, and M. D. Cave.** 2002. Diversity of *Campylobacter* isolates from retail poultry carcasses and from humans as demonstrated by pulsed-field gel electrophoresis. *J Food Prot* **65**:957-62.
11. **Donabedian, S., L. A. Thal, P. Bozigar, T. Zervos, E. Hershberger, and M. Zervos.** 2003. Antimicrobial resistance in swine and chickens fed virginiamycin for growth promotion. *J Microbiol Methods* **55**:739-43.
12. **Emborg, H. D., J. S. Andersen, A. M. Seyfarth, S. R. Andersen, J. Boel, and H. C. Wegener.** 2003. Relations between the occurrence of resistance to antimicrobial growth promoters among *Enterococcus faecium* isolated from broilers and broiler meat. *Int J Food Microbiol* **84**:273-84.
13. **Engberg, J., F. M. Aarestrup, D. E. Taylor, P. Gerner-Smidt, and I. Nachamkin.** 2001. Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: resistance mechanisms and trends in human isolates. *Emerg Infect Dis* **7**:24-34.
14. **Evans, M. C., and H. C. Wegener.** 2003. Antimicrobial growth promoters and *Salmonella* spp., *Campylobacter* spp. in poultry and swine, Denmark. *Emerg Infect Dis* **9**:489-92.
15. **Friedman, C., S. Reddy, M. Samual, R. Marcus, J. Bender, S. Desai, B. Shiferaw, D. Helfrick, M. Carter, B. Anderson, and M. Hoekstra.** 2000. Presented at the International Conference on Emerging Infectious Diseases, Atlanta, GA, July 16-19, 2000.
16. **Fuzihara, T. O., S. A. Fernandes, and B. D. Franco.** 2000. Prevalence and dissemination of *Salmonella* serotypes along the slaughtering process in Brazilian small poultry slaughterhouses. *J Food Prot* **63**:1749-53.
17. **Liesegang, A., and H. Tschape.** 2002. Modified pulsed-field gel electrophoresis method for DNA degradation-sensitive *Salmonella enterica* and *Escherichia coli* strains. *Int J Med Microbiol* **291**:645-8.
18. **Lipsitch, M., R. S. Singer, and B. R. Levin.** 2002. Antibiotics in agriculture: when is it time to close the barn door? *Proc Natl Acad Sci U S A* **99**:5752-4.
19. **Malorny, B., J. Hoorfar, C. Bunge, and R. Helmuth.** 2003. Multicenter Validation of the Analytical Accuracy of *Salmonella* PCR: towards an International Standard. *Appl Environ Microbiol* **69**:290-6.
20. **Marano, N. N., S. Rossiter, K. Stamey, K. Joyce, T. J. Barrett, L. K. Tollefson, and F. J. Angulo.** 2000. The National Antimicrobial Resistance

Monitoring System (NARMS) for enteric bacteria, 1996-1999: surveillance for action. *J Am Vet Med Assoc* **217**:1829-30.

21. **McAvoy, S.** 2000. Glickman announces national standards for organic food. USDA. [Online.] <http://www.usda.gov/news/releases/2000/12/0425.htm>. Accessed: April 14, 2004.
22. **National Committee for Clinical Laboratory Standards.** 2001. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals - approved standards M31-A. NCCLS, Wayne, PA.
23. **Rahn, K., S. A. De Grandis, R. C. Clarke, S. A. McEwen, J. E. Galan, C. Ginocchio, R. Curtiss, 3rd, and C. L. Gyles.** 1992. Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol Cell Probes* **6**:271-9.
24. **Romling, U., and B. Tummeler.** 2000. Achieving 100% typeability of *Pseudomonas aeruginosa* by pulsed-field gel electrophoresis. *J Clin Microbiol* **38**:464-5.
25. **Silbert, S., L. Boyken, R. J. Hollis, and M. A. Pfaller.** 2003. Improving typeability of multiple bacterial species using pulsed-field gel electrophoresis and thiourea. *Diagn Microbiol Infect Dis* **47**:619-21.
26. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* **33**:2233-9.
27. **Wegener, H. C.** 2003. Antibiotics in animal feed and their role in resistance development. *Curr Opin Microbiol* **6**:439-45.
28. **Witte, W.** 1998. Medical consequences of antibiotic use in agriculture. *Science* **279**:996-7.

