

ABSTRACT

Title of Thesis: CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* ISOLATED FROM ORGANIC RETAIL CHICKEN

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Listeria monocytogenes is an important foodborne pathogen. It is commonly found in the environment, frequently present in the gut of cattle, poultry, and pigs and can be transmitted to ready-to-eat foods as well as raw meat products. However, no data are available on the prevalence of *L. monocytogenes* in organic foods. In this study, 210 organic chickens collected from retail stores in the Washington DC area were examined for the presence of *Listeria* sp. using a modified Food and Drug Administration protocol developed to isolate the organism from meat products. Forty-eight organic chickens were positive for *L. monocytogenes*. The isolates were serotyped using PCR and subtyped by Pulsed-field gel electrophoresis (PFGE) to determine their genetic relatedness. The data

revealed that several *Listeria* sp. were present on raw retail chicken and *L. monocytogenes* serotypes associated with human listeriosis were also identified in the product.

CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* ISOLATED
FROM RETAIL ORGANIC CHICKEN

by

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Introduction

Listeria monocytogenes, was first reported in 1924 by E.G.D. Murray [54]. Murray isolated the organism, which caused monocytosis, from rabbits and guinea pigs. In 1929, Nyfeldt described the *Bacterium monocytogenes hominis* [57] as pathogenic to humans as well. It was not until the early 1980's that *L. monocytogenes* was first recognized as a food-borne pathogen [74].

Although today there is a clearer understanding of the organism and its relationship to other organisms, this has not always been the case. Throughout its history *Listeria* has been observed, studied and phylogenetically classified by numerous researchers. Because of the uncertainty of its phylogenetic position and its morphological similarity to the group of coryneform bacterium, names such as, *Corynebacterium parvulum* [79] and *Corynebacterium infantisepticum* [64] have been used to describe the organism.

It was not until the 1970s that its phylogenetic relationship, reinforcing its distinctiveness from the coryneform bacteria, was better understood. Its position was better clarified because of the development of numerical taxonomy, chemotaxonomy, DNA/DNA hybridization and rRNA sequencing techniques [70]. Currently the genus is taxonomically classified with its phylogenetic position being closely related to *Brochothrix* [20]. However its relationship with other low G+C% Gram-positive bacteria [26, 69, 87], as well as *Bacillus* and *Staphylococcus* still needs to be clarified.

Since the organism was first recognized, much information on its ecology, pathogenicity and the epidemiology of listeriosis has been revealed, yet the organism is still not completely understood nor is its presence in food under control.

Organism

Listeria is a gram-positive, non-sporulating, catalase positive, oxidase negative rod, which measures 0.5 μm in diameter and 1 - 2 μm in length. Gram stains show that the cells can be found in chains or as single rods. Growth of the organism on bacteriological media is enhanced by the presence of glucose or other fermentable sugars but is also dependent on the atmosphere and temperature in which they are grown. The organism can grow over a wide range of pHs (4.3-9.6), water activity (~ 0.83) and salt concentrations (up to 10 %) as well [83]. *Listeria* are aerobic, microaerophilic and facultatively anaerobic and can be cultured over a wide temperature range.

The organism has a growth temperature range of approximately 1°C - 45°C , [44], making it a psychrotroph and a mesophile. There are however, growth factors which are temperature dependent. For example, at $20\text{-}25^{\circ}\text{C}$ peritrichous flagella are formed and cause the organism to be motile, whereas at 37°C the organism is weakly or non-motile [29]. Additionally, its ability to not only survive but to grow as a psychrotroph at 4°C makes this pathogen unique from other commonly found food-borne pathogens which are usually inhibited from growth at refrigeration temperatures.

For many years the genus *Listeria* only contained one species, *L. monocytogenes*. Currently however, there are six recognized species including *L. monocytogenes*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*, and *L. grayi* [70, 93]. Although there are six distinct species they all have similar genetic homology which helps explain their similar phenotypic traits. Hemolysis as well as acid production are key characteristics in distinguishing among the species. At present only strains of *L. monocytogenes* are pathogenic to humans and animals, while *L. ivanovii* are only pathogenic in animals, particularly ruminants [95].

Virulence

The *L. monocytogenes* genome is approximately 3.0 Mb [53] (Genbank/EMBL accession number AL591824) and information on its sequence can be found at The Institute for Genomic Research (www.tigr.org). Virulence and virulence-like genes on *Listeria's* chromosome code for surface and secreted proteins as well as other regulators which help it to adapt to diverse environments and for expression of virulence traits (Table 1).

Table 1: *L. monocytogenes* genomics.

<i>L. monocytogenes</i>	
Size of chromosome (kb)	2,944,528
G+C content (%)	39
G+C content of protein-coding genes (%)	38
Total number of protein-coding genes	2853

Adapted from [35]

Further, species such as *L. innocua* lack genes which are essential for virulence. For example, a virulence gene such as one which codes for a surface protein and

plays a role in invasion is present in *L. monocytogenes* but is absent in *L. innocua* and this may help explain the different pathogenic potentials of different species [35, 45].

Listeria monocytogenes and *L. innocua* both contain a virulence gene cluster located on an 8.2 kb pathogenic island on its genome [45] which is regulated by the main positive regulatory factor A regulon (PrfA) [18]. The cluster is located between the *prs* and *ldh* genes on the chromosome [36] (Figure 1).

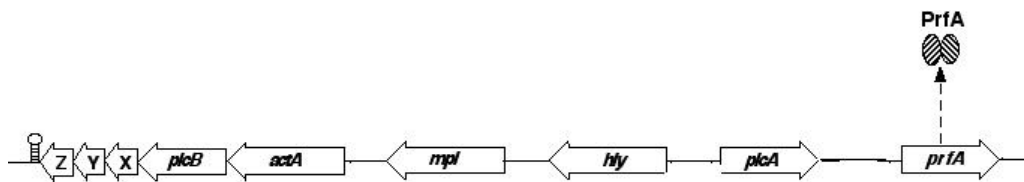


Figure 1: Virulence gene cluster located in *L. monocytogenes*.

Modified from <http://mcb.berkeley.edu/courses/mcb103/02PrfAModelSlide.gif>

This cluster is the only one known to date that is involved in the virulence of *Listeria*. It contains the majority of the known virulence genes which are involved in the invasion and intracellular cycle of the pathogen. The cluster encodes six genes, *prfA*, *plcA*, *hly*, *mpl*, *actA*, *plcB*, and three additional small open reading frames (orfs) X, Y, and Z downstream of *plcB* [46]. The PrfA gene is essential for the virulence of *L. monocytogenes*. It acts as the master regulator of virulence and virulence-like genes to varying degrees [19]. The remaining virulence genes on the virulence gene cluster result in the protein products of

listeriolysin O (LLO) by *hly*, a phosphatidylinositol-specific phospholipase C (PI-PLC) by *plcA*, a phosphatidylcholine-specific phospholipase C (PC-PLC) by *plcB*, a metalloprotease (*mpl*), an actin polymerization protein (ActA) by *actA*, and three genes, X, Y, and Z which functions are currently unknown.

Several other genes involved in virulence are located outside of but are still related to the gene cluster [46]. These genes which are involved in the production of surface proteins necessary for internalization of the pathogen to the host cell, include the *inlA*, *inlB* and *inlC* genes which code for internalin A, B, and C respectively as well as the *iap* virulence-like gene which codes for p60. However, it seems there are still other virulence and virulence-like genes which are either only partially regulated by or totally independent of PrfA [49, 84] (Table 2).

Table 2: The genes that play a role in the pathogenesis of *L. monocytogenes*. [68]

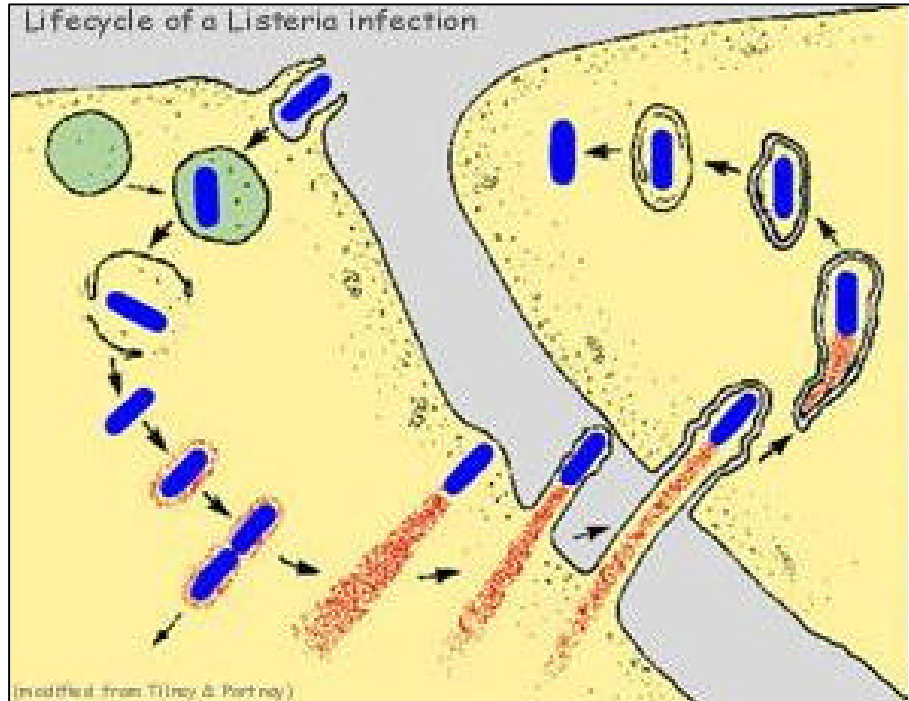
Gene		Function
<i>prfA</i>	positive regulatory factor A	transcriptional activator
<i>plcA</i>	phosphatidylinositol-specific phospholipase C	aids in escape from vacuoles
<i>plcB</i>	phosphatidylcholine-specific phospholipase C	aids in escape from vacuoles
<i>hlyA</i>	listeriolysin O	escape from vacuoles
<i>mpl</i>	zinc-dependent metalloprotease	maturation of PlcB
<i>actA</i>	actin-polymerizing protein	cell-to-cell spread
<i>inlA</i>	internalin A	internalization
<i>inlB</i>	internalin B	internalization

Listeria's pathogenicity is not only contributed by its existence as an intracellular pathogen but also to its ability to invade and replicate within a wide

range of mammalian cells. The rate of internalization is dependent on the cell type and may be mediated by at least one bacterial surface protein. The bacterial surface proteins *inlA* and *inlB* [10] are responsible for promoting the binding and internalization by either E-cadherin or the Met receptor tyrosine kinase and PI3-kinase activation respectively [21].

Once the organism is internalized a key virulence determining protein, LLO, as well as other secretory proteins PI-PLC and PC-PLC, aid the release of the bacteria from the vacuole which it resides in [31]. After cells have multiplied in the cytosol, an actin-based motility protein necessary for cell-to-cell spread, ActA is synthesized. The protein induces the polymerization of host actin filaments and allows the pathogen to propel itself into other cells as a type of pseudo-pod while evading the host's defenses [63]. This intracellular cycle and cell-to-cell spread is then continued onto the next cells to continue the infection. (Figure 2).

Figure 2: Intracellular invasion by *L. monocytogenes* [89].



Disease

Listeriosis is the disease caused by *L. monocytogenes* infections. *Listeria* is widely distributed in the environment and can also be found in the gastrointestinal tract of individuals who remain as asymptomatic carriers. This non-invasive listeriosis occurs in healthy adults but generally only amounts to gastrointestinal illness, fever, vomiting and diarrhea, where the degree of severity is dependent on the characteristics of the host and the organism's environment.

On the other hand, the more severe form of listeriosis is invasive listeriosis. The most common invasive listeriosis infections occur in children, the elderly, pregnant women and their fetuses and the immunocompromised. With the onset

of epidemics such as HIV/AIDS, there has been an increase in the size of the population at risk of morbidity and mortality due to this type of listeriosis [75]. The disease can manifest as septicemia, meningitis, meningoen­cephalitis or febrile gastroenteritis and can cause still births and abortions [93].

The infective dose has not yet been definitively determined but it may take less than 1000 cells to cause infection. However, this is dependent on the immunity of the infected individual and the strain of the organism. The incubation period can range from a few days to three weeks and may be preceded by gastrointestinal symptoms which manifest after approximately 12 hours incubation [52].

The rate of normal healthy adult infection from listeriosis is low. There are approximately 0.7 cases per 100,000 persons. However, the infection is more common in children at a rate of 10 cases per 100,000 person, and the elderly with 1.4 cases per 100,000 person [32]. Pregnant women are seventeen times more likely than healthy adults to acquire the infection [86].

Although listeriosis is considered a food-borne infection and most outbreaks are transmitted by food, there have been reports of large outbreaks attributed to other modes of transmission. For instance, a neonatal outbreak in Costa Rica involved the use of contaminated mineral oil for cleaning infants after delivery [77].

In addition the pathogens which cause neonatal bacterial meningitis in North America, *Listeria* is the third most common pathogen followed by group B

streptococcus and *E. coli* [23]. Fetuses can acquire the infection through the mother who has either colonized the organism in the gastrointestinal (GI) tract after consumption of contaminated foods or during childbirth if a mother is carrying *Listeria* in the GI or the perianal region, which in turn can contaminate the skin and respiratory tract of the child during birthing. Although cases unrelated to food do occur, foodborne transmission is the most common source of transimission of *Listeria* to humans.

In the U.S there are an estimated 76 million cases of food-borne illnesses each year. The incidences of listeriosis only average 2500 infections yearly but cause 500 fatalities [52]. Although the actual number of infections is low, a mortality rate, which can be as high as 20-30% regardless of antimicrobial treatment shows the danger that the presence of *Listeria* poses in foods. The mortality rate is considerably higher than the more common infections from other food-borne pathogens such as *Escherichia coli* O157:H7 (*E. coli*), *Campylobacter* spp. and *Salmonella* spp. [52] (Table 3).

Table 3: Comparison of the mortality rate among *L. monocytogenes* and other common food-borne pathogens [52].

Pathogen	Mortality Rate (%)	Hospitalization Rate (%)	Total Cases
<i>Campylobacter</i> spp	<1	17.3	~1.9 million
<i>E. coli</i> O157:H7	<1	3.0	~62,500
<i>Salmonella</i> spp.	<1	25.6	~1.3 million
<i>L. monocytogenes</i>	20.0	3.8	2500

There have been several sporadic and epidemic outbreaks worldwide implicating *Listeria* contaminated foods (Table 4) [1, 3, 4, 16, 27, 74, 81]. Foods which are denoted as ready to eat (RTE) foods (deli meats, salads etc.), unpasteurized dairy foods (cheese and milk), cured and raw meats (hot dogs, undercooked chicken), and items such as prepared seafood salads and even raw and unprocessed meats have been common foods implicated [75]. Although a look back at some initial recordings of the organism and their related outbreaks demonstrate that a food-to-human route of transmission was likely, it was not established until the 1980's [73]. There is still more to uncover concerning *Listeria's* relationship between environment, human and food.

Listeriosis is often treated with antimicrobials. The most favored treatment consists of using a combination of ampicillin and an aminoglycoside [42]. However the use of vancomycin in place of ampicillin is acceptable as well. Another treatment is the use of trimethoprim-sulfamethoxazol (TMP-SMZ) and rifampin. Cephalosporins, which are typically used for treatment of meningitis, are not effective for treating listeriosis because of *L. monocytogenes* resistance to this drug. However cephalosporins can be used in combination with ampicillin for listerial meningitis [75]. Overall, antimicrobial treatment against listeriosis can be slow and may even be untreatable or persistent [85].

However remaining vigilant about monitoring the pathogen is also important. This can be demonstrated by examining the way outbreaks are analyzed. PFGE patterns show which strains are likely responsible for an

outbreak by matching the genomic patterns from clinical samples to suspected foods which may be vehicles of transmission. With the cooperation and standardization of laboratories performing the analysis, this becomes a rapid method for determining the source of infection by sharing of information and perhaps limiting the number of infections and subsequent deaths [38].

Characterization of the Organism

Phenotyping and genotyping methods to discriminate bacterial strains are valuable tools which have different levels of discriminatory power. These methods are able to provide information on strains which may be responsible for an outbreak, or to identify the relationship between isolates implicated in an outbreak, and also to help determine a source of transmission for an outbreak.

Serotyping is based on the antigenic determinations expressed on the cell surface of the organism. These antigens are produced by lipoteichoic acids, membrane proteins, and extracellular organelles such as fimbriae and flagella [82]. Different strains of *L. monocytogenes* express different antigenic determinations, thus each strain can be serologically identified.

Listeria strains are separated based on flagellar (H) and somatic (O) antigens resulting in more than 13 serotypes [82, 38]. Genotypic analysis generally group *Listeria* into two main lineages, Lineage I and II while it is believed that there may be a third subgroup, Lineage III, as well.

Lineage I includes serotypes 1/2a, 1/2c, 3a, and 3c 1, Lineage II includes serotypes 1/2b, 3b, 4b, 4d and 4e while serotypes 4a and 4c have been grouped

with the third less common Lineage III [66, 37, 13]. The three serotypes which most commonly cause disease (> 95%) are 1/2a, 1/2b and 4b [60]. Although serotype 4b is most frequently implicated in foodborne diseases, it is serotype 1/2a which is most frequently isolated from foods [34].

Historically, serotyping of *Listeria* by antiserum has shown to be unreliable. A study by [76] and the World Health Organization (WHO), demonstrated that different laboratories using antiserum serotyping methods, either could not 100% correctly identify or 100% agree on the correct serotype of all the given isolates [76]. In addition, serotyping with antiserum has been shown to have less discriminating power than other methods.

For example, a Mismatch Amplification Mutation Assay (MAMA) using mismatched PCR primers targeting different sites in the *hly* gene was developed to rapidly screen *L. monocytogenes* isolates into their respective phylogenetic divisions [43]. Other methods such as one using PCR primers which target select sequences in the four major serotypes to adequately differentiate them have also been recently developed. The results obtained with the PCR primers agree significantly enough with the traditional slide agglutination method [9] that perhaps this can one day be a standardized method.

The discriminatory power of phenotypic and genotypic methods have also been investigated. In one study, phage typing was the most discriminatory of the methods tested followed by REA, MEE, and ribotyping when looking at differences based on “O” serotype discrimination. However, REA best

discriminates for serotype 1 and phage typing was best for discriminating serotype 4 [55]. However, there were studies which noticed that the discriminatory power of methods such as MEE and ribotyping could not adequately differentiate between closely related serotypes such as 1/2b and 4b [37].

Other methods to differentiate *L. monocytogenes* phenotypes, such as antimicrobial susceptibility are of limited use currently. At present there are no standards or standard guidelines which have been established for the organism. *L. monocytogenes* susceptibility to antimicrobials has also been relatively constant for several years, although there has been some evidence found that there are plasmids present which confer resistance to chloramphenicol, macrolides and tetracyclines [40, 65].

Although phenotypic or conventional methods such as serotyping, bacteriophage typing, bacteriocin typing, and antimicrobial susceptibility have been used, with the development of molecular techniques some of those methods may have limited use in the future.

Some molecular methods that are commonly used include Multilocus Enzyme Electrophoresis (MEE), chromosomal DNA Restriction Endonuclease Analysis (REA) as well as Restriction Fragment Length Polymorphism (RFLP), Ribotyping and PFGE. The advantages to using each of these molecular techniques for genetic analysis vary. Several researchers along with those at the WHO's Multicenter *Listeria monocytogenes* subtyping study have compared the

various molecular methods to determine which have the most discriminatory power.

Although MEE is a powerful tool for population genetic, taxonomic and evolutionary studies, its ability to discriminate subtypes for epidemiological investigations is limited [60, 38]. Further, REA is universally applicable and sensitive because an entire genome can be evaluated easily and cost effectively, however its complex profiles, which may consist of hundreds of bands, is not practical to use epidemiologically [33, 38]. Ribotyping and RFLP have also been evaluated in their usefulness for epidemiological purposes. Ribotyping has been shown to be not as discriminatory as REA or MEE in subtyping *L. monocytogenes* [55, 38].

PFGE, another powerful subtyping technique, is considered the ‘gold’ standard for subtyping *L. monocytogenes*. PFGE takes advantage of restriction enzymes that cut genomic DNA infrequently which in turn produces simple profiles that can be analyzed by computer based methods. This allows easy, fast and simplified comparison of strains. This is extremely important when investigating food related outbreaks [39]. Strains suspected in outbreaks can be rapidly compared to those stored in a database which contains known epidemiologically significant strains.

Additional researchers demonstrated the usefulness of PFGE in discriminating between closely related 4b strains [12]. PFGE has since been shown to be applicable for use in typing strains involved in outbreaks of listeriosis

[15]. An additional study done by Brosch [14] in collaboration with WHO reinforced the discriminatory and reproducibility of using PFGE in subtyping compared to the other methods which are less discriminatory and not satisfactorily subtyped especially for non-diverse serotypes such as the 4b group.

PFGE uses an infrequent restriction endonuclease such as *AscI* to digest the *Listeria* genome into large fragments (> 40 kb). By using constant current pulsed field homogenous electric field (CHEF) PFGE these larger fragments are then subjected to two opposing electrical gradients of a specified angle in order to allow the DNA to 'zig-zag' through the agarose gel. The DNA is then visualized using ultraviolet light after they have been stained and destained with ethidium bromide. The fragments are seen as bands and their patterns can be compared with other patterns, such as those from other outbreaks or clinical samples, in order to help determine the epidemiology or origin of the strain.

PulseNet, the National Molecular Subtyping Network for Foodborne Disease Surveillance (www.cdc.gov/pulsnet), is made up of a national network of public health and food regulatory laboratories in the U.S. These labs use standardized PFGE protocols to subtype bacteria which may be implicated in a food-borne outbreak. This network of laboratories in collaboration with the Centers for Disease Control (CDC) can use computers to rapidly compare PFGE patterns of isolates from patients and implicated samples from across the U.S. in order to determine the source of transmission. The PulseNet network allows for a

rapid and standardized medium for public health officials to rapidly determine the epidemiology of an infection and to stop it from spreading.

There has been a concerted effort to develop rapid protocols for organisms under the surveillance of PulseNet. Currently, protocols for *E. coli* O157:H7, *Salmonella* spp. and *Shigella* spp. have been developed [30]. In 2001 researchers at the CDC, developed a rapid standardized protocol for subtyping *Listeria* [39]. The result is a 30 hour protocol which has the same discriminatory power as a previously used multi-day protocol.

***Listeria* and Food**

Because *Listeria* is ubiquitous in nature, its route of transmission was not always definitively categorized. Since the 1980's, it has been conclusively determined by epidemiologic and laboratory surveillance of contaminated cheese and raw vegetable outbreaks, that listeriosis is indeed transmitted by consumption of contaminated foods [74]. Although *Listeria's* relationship between the environment, animals, humans, and food is still not absolutely clear, this categorization of *Listeria* as a food-borne pathogen as well as its potential to harm brought the organism to the forefront of food safety and regulatory issues.

There have been several sporadic and epidemic outbreaks worldwide implicating *Listeria* contaminated foods (Table 4) [1, 3, 4, 16, 27, 74, 81].

Table 4: Summary of historical listeriosis outbreaks and their vehicle of transmission.

Vehicle of Infection	Year	Location	Number Infected	Mortality Rate (%)	Ref
Coleslaw	1981	Nova Scotia	41	34	74
Turkey, chicken deli meats	2002	Northeastern US	46	22	1
Hot dogs	1999	United States	101	21	3
Mexican-Style Cheese	1985	Western US	142	34	4
Soft Cheese	1983-1984	Switzerland	57	32	16
Pasteurized Milk	1983	New England	49	29	27
Unknown	1989	Pennsylvania	36	44	81

Foods which are denoted as ready to eat (RTE) foods (deli meats, salads etc.), unpasteurized dairy foods (cheese and milk), cured meats (hot dogs, undercooked chicken), and items such as prepared seafood salads and even raw and unprocessed meats have been common foods implicated [75]. Since there is still more to uncover concerning *Listeria's* relationship between environment, human and food, it is necessary to emphasize the importance of prevention. This has prompted the U. S. Food and Drug Administration (FDA), the Food Safety and Inspection Service (FSIS), and the U.S. Department of Agriculture (USDA) to mandate a level of 'zero tolerance' (no detectable level) for *Listeria* in RTE foods.

[88]. Other European nations are aware of the risk from listeriosis and have implemented risk-based standards for keeping *Listeria* out of foods.

Some noteworthy outbreaks which occurred in the United States include the following. In the fall of 2002, the largest recall in United States history occurred because of *Listeria* contamination. This recent and important outbreak affected multiple states encompassing the northeastern U.S. and involved a recall of approximately 27.4 million pounds of fresh and frozen turkey and chicken deli meats. There were 46 confirmed cases of infections, with seven deaths and three still births reported across eight northwestern states. The overall mortality rate from this outbreak reached 22%, while 6.5% of those who died were new born infants [1].

In addition, several outbreaks have been linked to soft-style cheeses such as those which occurred in 1985 [4] and another in 2000-01 [2]. The former outbreak, which covered a 7.5 month epidemic period involved 142 listeriosis cases resulting in 48 deaths or a 33.8% mortality rate. Thirty of the deaths were fetal or neonatal deaths. The latter outbreak resulted in 12 infections. All the patients were pregnant women except for one post-partum woman and another immuno-compromised elderly man. The infections resulted in five stillbirths, three premature births and two infected newborns [21].

This also reinforces the need to continue surveillance programs such as PulseNet and CDC's Foodborne Diseases Active Surveillance Network (FoodNet). These programs allow for rapid epidemiological investigations to be performed.

By sharing of databases with local, state and federal public health laboratories, potentially serious outbreaks can be timely identified and foods implicated recalled as quickly as possible.

Food safety programs such as the Hazard Analysis Critical Control Point (HACCP) in addition to consumer education programs such as FIGHT - BAC® and Thermy® (FSIS, USDA) are also in place. The former educate food producers and food handlers while the latter educate consumers about the importance of contamination and food safety at home. Although there is a need for food safety at the production level, the consumers can help lower the risk of foodborne infections through proper education from understanding the need to chill at proper temperatures, separate raw meats and ready to eat foods as well as cooking to proper temperatures.

Although on-going efforts and new efforts are continually being made to eradicate food-borne pathogens from the food supply, they still persist and in some instances have adapted to the pressures used to eradicate them. Several case studies done by the CDC [62, 78, 80] identified undercooked chicken as a high-risk vehicle of transmission. Although the role that *Listeria* plays in raw and undercooked chicken is still somewhat unclear, because with proper heating and food handling *Listeria* is often killed in raw foods and meats.

Food survey studies conducted in other countries and in the U.S. also show a prevalence in conventional chickens, although no data are available on organic chickens. For example, in 2001 a study done in Spain showed that *L.*

monocytogenes was found on 15% of the chicken carcasses tested [17]. Other studies have indicated that the rate of recovery of *L. monocytogenes* in raw chicken was 8 out of 70 (11.4%) total samples [94] and 34 of 58 (59%) [48] (Table 5).

Table 5: Prevalence of *L. monocytogenes* on chicken carcasses from other studies performed worldwide.

Country	Prevalence	Reference
Spain	32%	17
Norway	61%	71
United Kingdom	60%	61
Spain	64%	28
United States	23%	6
Belgium and France	10-15%	48
Ireland	59%	91

The pathogen remains problematic for the food industry because contamination often occurs during processing or post processing. With the modernization and centralization of food processing, *Listeria's* ability to survive and multiply has prevailed even with improved food preservation methods. Therefore in order to control the organism, it is necessary to minimize the potential points of entry for the pathogen.

Over the years, a pattern has been seen which indicates that the initial point of contamination begins in the food processing environment. This trend is also evident in the slaughter houses of raw meats such as pork and poultry. Prior to slaughter, contamination of live animals with the pathogen is low. However once the animal enters the processing facility and is slaughtered, the prevalence in the environment becomes high. In surveys of a chilling-cutting area of several

processing plants, post-slaughtered meats demonstrated a 70-100% increase in prevalence of the *Listeria* organism [92]. Similar trends of post-slaughter contamination in the processing plant are seen with poultry and turkey slaughterhouses [22, 58, 59], and smoked fish plants [5, 56, 72].

It also seems that the packaging of poultry products is important. *Listeria's* survival can be dependent on the ability of a packing method to suppress other organisms. Wimpfheimer [96] looked at the behavior of *Listeria* in different packing and storage conditions. There was an increase in the organism when raw chickens were packaged in a micro-aerophilic or an aerobic atmosphere and left in 4°C for an extended storage time. However there was a decrease in the number of spoilage organisms in the same package under microaerophilic conditions and a similar increase to *Listeria* in the aerobic packaging [96].

Although there have been data collected on foods both processed and raw, there has been a lack of data on a fast growing segment of the food industry, organic foods. The growth of the organic food industry has increased 20 - 25% in the U.S. over the last seven years. This has amounted to retail sales of \$3.5 billion in 1996 up from \$1 billion in 1991. There has also been an increase in foreign markets [24]. Consumers and farmers have been increasingly interested in organic food products because organic foods are considered natural and healthy, although the health benefits as well as the food safety risks have not been clarified and much work still needs to be done.

Organic farming emphasizes the use of renewable resources to produce foods. Soil and water are also conserved to improve the quality of the environment. Conventional pesticides, fertilizers or other synthetic ingredients are also not allowed. Livestock animals must be given access to outdoor free range and be fed organic feed. In addition, they are not given any antibiotics for either growth promotion or as treatment for any disease.

Because of the lack in data and microbiological impact with the way organic farming is done, it is interesting to look at the effect of organisms such as *Listeria* in this new type of environment. Organic livestock production may increase the risk of microbial contamination and thus foodborne illness, due to outdoor production and complete prohibition of antibiotic use. However, there is a paucity of data with regards to the microbiological safety of organic food products. The objective of this research was to determine the prevalence of *L. monocytogenes* in retail organic chickens, and to characterize the *L. monocytogenes* isolates and examine their genetic relatedness using PCR and PFGE.

Materials and Methods

Sample Collection and Preparation

Fresh organic chicken carcasses (n=210) were purchased from three specialty food and/or organic food markets in the Greater Washington DC area, including suburban Maryland. Overall four different sample brands were selected depending on which brands were available at the time of purchase in each store. In order to maintain a database of the isolates, each store and chicken sample was assigned an identifying number. The sampling took place over the course of eight months of one year from September 2002 to August 2003. Sampling occurred at the beginning of each week on a bi-monthly period.

In addition, conventional chickens (n=61) were also purchased in the last three months of the study (June 2003 – August 2003) for other purposes. These were obtained from three markets in the Greater Washington DC area and included six different brands.

On each sampling day, the chickens were aseptically removed from their store package and placed in a plastic bag containing 400 ml of sterilized buffered peptone water (Difco Laboratories, Detroit, MI). The rinse solution was manually massaged for at least 60 seconds and then used for the isolation of *Listeria* spp.

Bacterial Isolation

The protocol used to isolate *Listeria* spp. from conventional and organic chicken was a modified version of the FDA's Bacteriological Analytical Manual (BAM) [41]. Culturing of the *Listeria* spp. included 25 ml of the rinse being used

as a pre-enrichment broth by incubating for 4 hours at 30°C and shaking at 100 rpm in an orbital shaking incubator (Forma Scientific, Marietta, OH).

After four hours, the selective agents acriflavin HCL, used to inhibit Gram-positive cocci, (Sigma, St. Louis, MO) and nalidixic acid which inhibits Gram-negative bacteria through interference with DNA gyrase (Donnelly, 1999) (Sigma, St. Louis, MO) were added to the pre-enrichment as 0.5% w/v solutions in distilled water at concentrations of 0.455 ml acriflavin (Sigma, St. Louis, MO) and 1.8 ml nalidixic acid (Sigma, St. Louis, MO) to 225 ml enrichment broth [41].

Culturing media Oxford (OXA) (Oxoid, Hampton, England) and Palcam (PAL) (Difco, Detroit, MI) selective media were used for the isolation of *Listeria spp.* After 24 hours, 100 ul of the enrichment broth was spread plate onto OXA and PAL media and incubated for 24 hours at 35°C. Two to three colonies that exhibited a black halo due to the hydrolysis of esculin and ferric ammonium citrate differential agents [25] were streaked onto Tryptic Soy Agar with 0.6% yeast (TSAYE). The enrichment broths were re-incubated at 30°C, 100 rpm for an additional 24 hours. Although Gram-negative and most unwanted Gram-positive bacteria are completely inhibited, organisms such as *Enterococci* can grow poorly and may exhibit a weak aesculin reaction, typically after 40 hours incubation on these media. Some *Staphylococci* can also grow as aesculin negative colonies as well [11]. Additionally, on PAL media *Listeria* exhibits as gray-green colonies with a black precipitate after incubation at 35°C for 24-48 hours under aerobic or microaerophilic conditions. Mannitol fermenting colonies from organisms such

as *Staphylococcus* may grow and appear yellow with a yellow halo on this media. [11].

Negative OXA and PAL plates after 24 hours were re-incubated for 48 hours at 35°C. Also, an additional 100 ul were spread onto new OXA and PAL for those that did not have positive growth and were incubated for 24 hours, checked and streaked onto TSAYE if there was positive growth. Negative or no growth plates were re-incubated for an additional 24 hours then examined and treated the same as previous samples. Overall, both 24 and 48 hour enrichment broths were spread onto both OXA and PAL and incubated for a total of 24 or 48 hours.

Isolation Confirmation

PCR primers targeting the hemolysin (*hly*) gene were used for confirmation as previously described [47]. Briefly, the reaction was done under the following thermocycling conditions in a GeneAmp PCR System 9700 (PE Applied Biosystems); 60°C, 60 secs; (annealing), 94°C, 60 secs; (denaturing), 72°C, 60 secs (extension) for a total of 30 cycles using the sequences (f)5'CGGAGGTTCCGCAAAAGATG-3' and (r)5'CCTCCAGAGTGATCGATGTT-3'.

The templates were stored in 50/50 BHI/glycerol at -80°C until used. They were streaked onto TSAYE and colonies were swabbed into sterile water and lysed by boiling. The PCR reaction mixture contained the following concentrations of components for a total volume of 25 ul: 1X PCR buffer, 2.0mM

each dNTP, 2.0mM MgCl₂, the primers (f) 0.98 uM and (r) 0.74 uM and finally the enzyme *Taq* DNA polymerase 0.3 U/ul. The PCR products were visualized on a 1.5% agarose gel. The expected size of the PCR product was 234 bp.

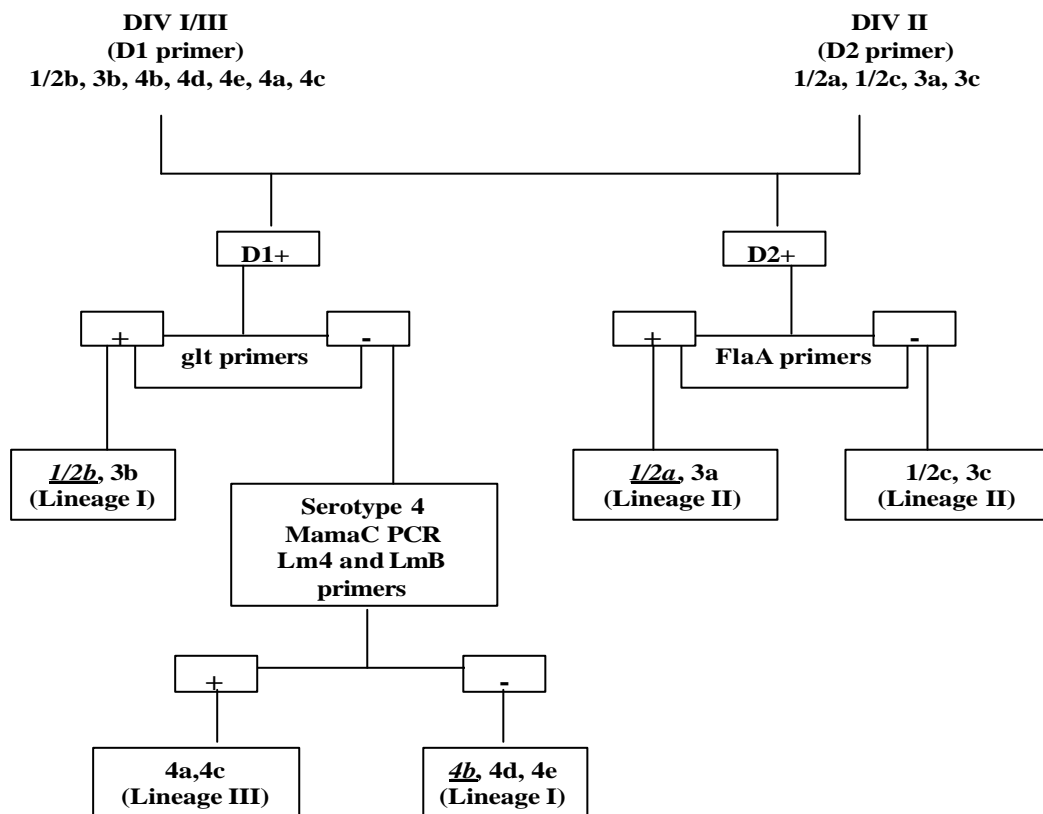
Confirmation of the isolates were also done with the Bio-Rad Rapid L. mono pre-packaged plates (BioRad, Hercules, CA). Plates were incubated at 35°C for 24 hours and results determined by the ability of the organism to ferment xylose and detect phospholipases. Strain ATCC # 15313 (UMD # 485) was used as a positive control. Seventy-four *L. monocytogenes* samples, 26 from conventional chickens and 48 from organic chickens were serotyped and subtyped. In addition, 10 isolates which could not be definitively confirmed using the RLM plates were further analyzed along with the 74 *L. monocytogenes* samples.

Serotyping By PCR

Isolates were serotyped using a PCR method previously described by Borucki [9]. The primers used to serotype were based on division specificity. All isolates were tested with division I/III (D1) primers CGATATTTTATCTACTTTGTCA(f) and TTGCTCCAAAGCAGGGCAT(r) and division II (D2) primers GCGGAGAAAGCTATCGCA(f) and TTGTTCAAACATAGGGCTA(r) (Table 6). The designs of these initial division primers were based on sequence differences of ferrous iron transport proteins between the two divisions from previous microarray research [9]. Additionally all D1 positive isolates were further tested with primer set *glt* AAAGTGAGTTCTTACGAGATTT(f) and AATTAGGAAATCGACCTTCT(r)

which were designed with sequences from the *gltA-gltB* flanking region in 1/2b serotype. Therefore negative *glt* samples were then tested with mismatched amplification mutation assay (MAMA) PCR primers previously designed [43] to differentiate the 4 serotypes. All D2 positive isolates were further tested using *FlaA* primers TTACTAGATCAAACCTGCTCC(f) and AAGAAAAGCCCCTCGTCC(r) which encodes the flagellin protein and is conserved between serotypes 4b and 1/2a [9] (Figure 3).

Figure 3: Overview of PCR primers used to serotype.



The PCR amplification conditions were as follows. DNA templates were made from stock cultures of the isolates kept in 50/50 BHI/glycerol at -80°C .

They were grown on brain heart infusion (BHI) agar at 37°C overnight (16-18 hrs). The cultures were then swabbed and resuspended in 500ul of 1X TE buffer. The samples were vortexed and boiled for 10 minutes. After boiling lysis, they were centrifuged and the supernatant collected in a new 1.5 ml eppendorf tube and stored at -20°C until needed. The amplification conditions depended on which primers were being used. For all the reaction mixtures, 1X PCR Buffer, 20 mM MgCl₂, 20 mM of dNTP each, 0.2 U Taq polymerase, 25 pmol/ul of primer, and 2 ul of DNA template were used for a final 25ul total volume. The PCR cycling condition performed in a GeneAmp PCR System 9700 (PE Applied Biosystems) were as follows: 95°C for 3 mins, 95°C for 25 cycles (D1, D2, and glt), or 95°C for 30 cycles (FlaA) for 30 secs, 75°C for 60 secs. and a final step of 72°C for 10 mins post-cycle. Gels ranged from 1.2-2.0% depending on which primers were used (Table 6).

Table 6: Summary of PCR primers used to serotype.

Primer Set	Forward Primer Reverse Primer	Anneal Temp(° C)	Product (bp)
D1 ^a	CGATATTTTATCTACTTTGTCA TTGCTCCAAAGCAGGGCAT	59	214
Gl ^b	AAAGTGAGTTCTTACGAGATTT AATTAGGAAATCGACCTTCT	45	483
Mama ^c	CAGTTGCAAGCGCTTGGAGT GTAAGTCTCCGAGGTTGCAA	55	268
D2 ^a	GCGGAGAAAGCTATCGCA TTGTTCAAACATAGGGCTA	59	140
FlaA ^b	TTACTAGATCAAACCTGCTCC AAGAAAAGCCCCTCGTCC	54	538

^a 2% agarose gel

^b 1.2% agarose gel

^c 1.5% agarose gel

L. monocytogenes strains ATCC# 19114 (UMD #479) serotype 4a, ATCC# 15313 (UMD #485) serotype 1/2a, and ATCC# 13932 (UMD #492), serotype 4b, were used as positive controls.

PFGE Analysis

Plug Preparation

Bacterial strains were grown on BHI agar at 37°C for 16-18 hours. Falcon tubes #2057 (Becton Dickinson, Franklin Lakes, NJ) were used to adjust cells in 3 ml of 0.01M TE (pH 8) to a cell density of 0.79-0.81 OD with a Dade Microscan turbidity meter (Baxter Diagnostics, Inc. McGraw Park, Ill.). Two hundred forty microliters of the cell suspension was then placed in 1.5 ml eppendorf tubes and 60 ul of 10 mg/ml lysozyme was added. The tube was then incubated in a 37°C water bath for 10 mins. The cell suspension was then added

to 3 ul of ProteinaseK (PK) (Gibco BRL) in a clean 1.5 ml eppendorf tube. Seakem gold agar with 1% SDS, heated and stored in a 54°C waterbath was added to the tube for a total volume of 600 ul. Plugs were made in triplicate using BioRad disposable plug molds (BioRad, Hercules, CA). Once cooled, the plugs were placed in 4 ml of cell suspension buffer (50mM Tris, 50mM EDTA [pH 8.0], 1% sarcosine) and incubated for 2 hours in a 54°C water bath with 200 rpm shaking. The plugs were then washed with 15 ml of sterile water for 15 minutes, and 15 ml of 0.01M TE four times for 15 minutes each. The water and TE washes were then repeated one final time. Plugs were stored in 0.01M TE at 4°C or digested immediately.

Plug Digestion

Digestion was performed using the *AscI* restriction enzyme according to the manufacturer's instructions. The plugs were first cut to 0.2mm and placed in 1X buffer for a total volume of 200 ul. They were incubated for 10 minutes in a 37°C waterbath. Then the preincubation buffer was removed and the restriction incubation mixture containing 2.5 U of *AscI* was added and incubated in a 37°C waterbath for a minimum of three hours. *Salmonella Braenderup* was used as a control and was digested with 5U of *XbaI* according to manufacturer's instructions in a 37°C waterbath for a minimum of three hours.

Electrophoretic Conditions

Once restriction was completed, the plugs were dried of any excess restriction mixture from the edges and placed on a comb and allowed to air dry

for approximately five minutes. The *S. Braenderup* controls were placed on the comb in the one, eight and 15 positions. A 1% of SeaKem gold agarose gel was cast around the comb and the wells were backfilled once the agar solidified and the comb was removed. The gel was run in a BioRad CHEF DR-III electrophoresis apparatus for 20 hours at a 120° angle, 6.0volt/centimer gradient and a linear ramp factor. The cooler was set for 14°C and initial and final switches were set for 4 and 40 seconds respectively.

Gel Visualization

The gel was stained with [10mg/ml] of EtBr (Invitrogen, Carlsbad, CA) for 30 minutes and destained one to two times with sterile water for 20-30 minutes each after which it was visualized in a GelDoc 1000 (BioRad, Hercules, CA). The PFGE data were analyzed using BioNumerics software (Applied Math, Texas USA) at the Center of Veterinary Medicine – FDA to generate a dendrogram.

Results

Prevalence of *Listeria* spp. on organic and conventional chicken

A total of 210 organic chicken samples were collected from three retail stores in the Greater Metro Washington DC area from September 2002 to August 2003. Ninety-five chickens were from store X, 34 from store Y and 80 from store Z. *L. monocytogenes* was isolated from 48 (22.9%) of the samples. In addition to *L. monocytogenes*, *L. ivanovii* was found in 16 (7.6%) organic chickens. *L. innocua* was also isolated from 17 (8.1%) organic chickens. Four (1.9%) organic chickens were positive with *L. welshimeri*. The other two *Listeria* species were not present in any of the samples using the RLM confirmation method.

An additional 61 conventional chickens were also available for analysis. These samples were obtained for other isolation projects in the laboratory and the initial carcass rinse was prepared in an identical manner as the organic chicken rinse. Therefore they were included in the isolation analysis in order to give an insight to the prevalence of *Listeria* spp. on conventional chickens.

The conventional chickens were purchased over the course of three months from June 2003 through August 2003. Twenty-six (42.6%) of the chickens were from store O, 23 (37.7%) were from store P and 12 (19.7%) were from store Q. The prevalence rates show that *L. monocytogenes* was found on 26 (42.6%) chickens, while *L. welshimeri* was found on one (1.6%) and *L. innocua* on 18 (29.5%) of the 61 chickens.

Prevalence of *L. monocytogenes* on organic chicken

L. monocytogenes was isolated from forty-eight of the 210 organic chickens at a prevalence rate of 22.9%. Twenty-one (43.8%) of the chickens positive for *L. monocytogenes* were from Store X, 21 (43.8%) were from Store Y and six (12.5%) were from Store Z. Of the four organic brands tested 11 (22.9%) of the chickens positive for *L. monocytogenes* were from Brand A, 18 (37.5%) were from Brand B, 14 (29.2%) were from Brand C and five (10.4%) were from Brand D (Table 7). Overall there were a total of 153 *L. monocytogenes* isolates from the 48 chicken samples.

Prevalence of *L. monocytogenes* on conventional chicken

L. monocytogenes was isolated from 26 (42.6%) of the 61 chickens. Store O had 11 (42.3%) positive samples, Store P had 13 (50.0%) positive samples and Store Q had 2 (7.7%) of the remaining positive samples. Conventional brands E, F, I, and J had no positive isolates, while brand G and H had 19 (73.1%) and 7 (26.9%) of the positive isolates respectively. Overall there were a total of 90 *L. monocytogenes* isolates from the 26 chickens (Table 7).

Table 7: Summary of *L.monocytogenes* results for organic and conventional chickens by category.

	Organic	Conventional
	# positive (%)	# positive (%)
Store X	21 (43.8)	
Y	21 (42.8)	
Z	6 (12.5)	
O		11 (42.3)
P		13 (50.0)
Q		2 (7.7)
Brand A	11 (22.9)	
B	18 (37.5)	
C	14 (29.2)	
D	5 (10.4)	
G		19 (73.1)
H		7 (26.9)
Other		0 (0.0)
Totals	48 (22.9)	26 (42.6)

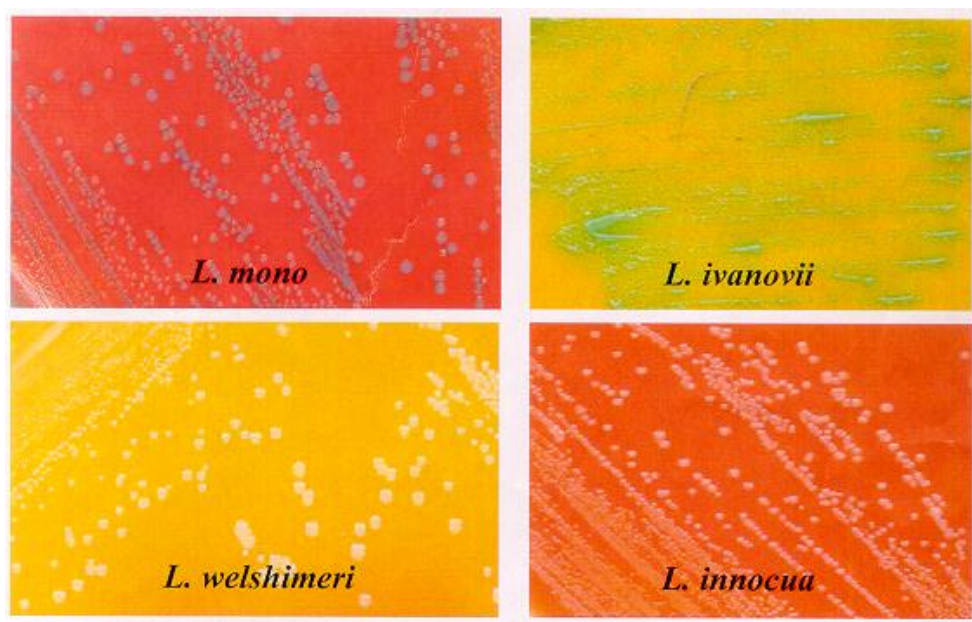
Confirmation using RLM plates

RLM plates are able to differentiate *Listeria* species based on their biochemical composition. *L. monocytogenes* and *L. innocua* are not able to ferment xylose. Therefore they do not exhibit a yellow halo around colonies unlike species such as *L. ivanovii* and *L. welshimeri* which can ferment xylose and do exhibit a yellow halo around colonies. Typically colonies of *L. monocytogenes* and *L. innocua* form characteristic blue-green colonies due to the media's ability to detect phospholipases. Those isolates which do not contain phospholipases exhibit as white colonies (Figure 4).

In addition to the 74 isolates to be serotyped and subtyped, there were 10 additional isolates included in further analysis because the Rapid L. mono plate

was not definitively able to confirm them to be *L. monocytogenes*. After serotyping and subtyping analysis it was determined that five were not *L. monocytogenes*, while none of the remaining five positive *L. monocytogenes* samples were from any chicken sample not already included in the original 74 sample analysis.

Figure 4: Confirmation of *Listeria* spp. on Rapid L.mono plates.

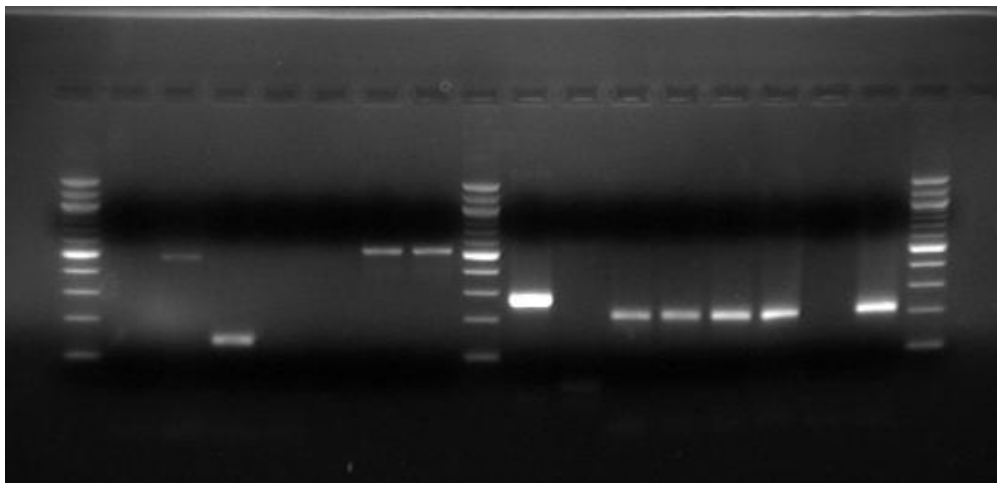


Serotyping

The PCR primers used to serotype both organic and conventional chickens resulted in 63 (85.1%) division I/III positive isolates and 11 (14.9%) division II positive isolates. D1 positive isolates were further tested using the *glt* primer set,

while D2 positive isolates were further tested with the *FlaA* primer set. Sixteen (25.4%) of the D1 isolates were positive with the *glt* primers and were further tested using the MAMA primers. The MAMA primer set resulted in no positive isolates except those of the positive control. Therefore the negative results determined that the serotype for those isolates were the 4b serotype. Ten (91%) of the D2 primers were positive when tested with the FlaA primers and therefore determined to be a 1/2a serotype (Figure 5).

Figure 5: Example of all positive results for each of the serotype primers.



Lanes 1, 8, and 18: 100bp molecular weight marker
Lanes 2-3: (-)Glt (UMD#479) and (+) Glt (433)
Lanes 4-5: (+)D2 (22) and (-) D2 (319)
Lanes 6-8: (-) FlaA (22) and (+) FlaA (485 and 415)
Lanes 10-11: (+) Mama-PCR (479) and (-) Mama-PCR (88)
Lanes 12-18: (+) D1 (12, 163, 167, 335) and (-) D1 (309)

Overall serotype distribution showed that ten (13.5%) of the 74 isolates were serotype 1/2a, while 47 (63.5%) were serotype 4b, 16 (21.6%) were serotype 1/2b and only one (1.4%) isolate was a 1/2c serotype.

For the 48 organic isolates the majority were of serotype 4b (75.0%). The remaining serotypes that were found included 1/2a (16.7%), 1/2b (6.3%) and 1/2c (2.1%) (Table 8).

Table 8: Summary of serotypes in organic chicken

Serotype	%(#)
1/2a	16.7(8)
4b	75.0(36)
1/2b	6.3(3)
Other (1/2c)	2.1(1)

For the conventional chickens there were only three serotypes present with half of the isolates belonging to the 1/2b serotypes. The remaining isolates were either a 4b or a 1/2a serotype. Of the 26 isolates, 13 (50.0%) were serotype 1/2b, 11 (42.3%) were 4b, while serotype 1/2a was only represented by two (7.7%) isolates (Table 9).

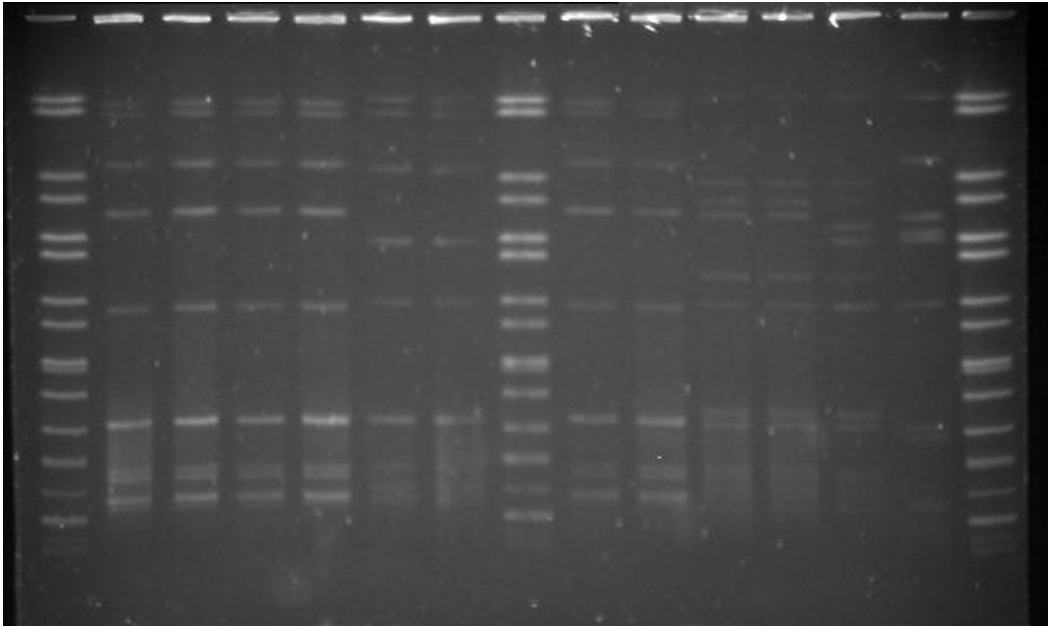
Table 9: Summary of serotypes in conventional chicken

Serotype	%(#)
1/2a	7.7(2)
4b	42.3(11)
1/2b	50.0(13)

Subtyping

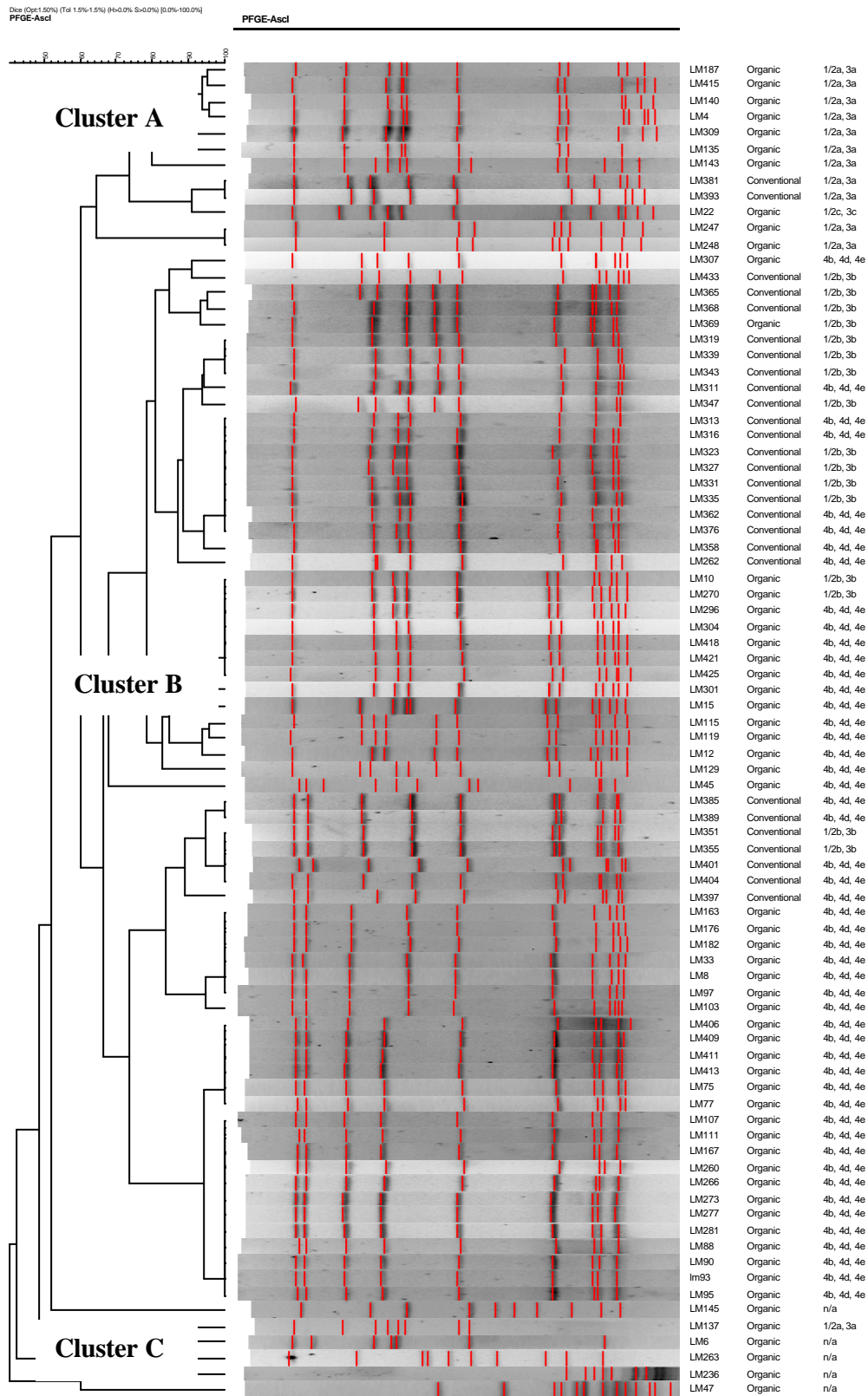
All isolates were characterized with the *AscI* restriction endonuclease. (Figure 6) From the analysis done with BioNumerics (Applied Maths, TX), a dendrogram exhibiting genetic relatedness was generated. There were three distinct clusters (A, B, and C) which were evident. The first group belong to those isolates that were the 1/2a and 1/2c serotype. The next contained the 4b and 1/2b serotypes and lastly the third cluster C contained the isolates which were determined not to be *L. monocytogenes*. There did not seem to be any distinct or clear pattern observed regarding the separation of the conventional and organic isolate patterns.

Figure 6: Example of PFGE patterns from some of the samples examined



Cluster A contained the 1/2a and 1/2c (lineage I) serotype for a total of 11 samples, both organic (9) and conventional (2), with nine different PFGE patterns. This cluster also contained the single 1/2c isolate which is also in lineage I. The 1/2b patterns of lineage II were all in the second cluster B and exhibited eight different patterns over 16 conventional (3) and organic (13) samples. The 4b serotype, also in lineage II, contained 20 different patterns over 47 organic (36) and conventional (11) chickens. In addition, there were two patterns that were identical across the 1/2b and 4b serotypes (Figure 7).

Figure 7: Dendrogram of PFGE Isolates



Discussion

A growing market in the food industry is the organic foods market. Not only have trends in the last seven years indicated a tremendous growth in new products and consumer interest but also in economic growth. The strict guidelines mandated by the USDA for animals grown as organic meats include not using antimicrobials for either growth promotion or for disease treatment. In addition, animals must be given free outdoor range and no fertilizers or pesticides can be used in the organic feed because one of the major tenets of organic farming includes the conservation of the environment. This gives the perception that organic foods may be healthier and safer, although there is a lack of data on the prevalence of contamination from microbes to help support these perceptions.

This study, which was conducted over the course of one year, from the end of September 2002 to the end of August 2003, indicates that there is a prevalence of *Listeria* spp. on organic and conventional chicken. The majority of the *L. monocytogenes* isolates belong to the 4b serotype, however serotype 1/2a, 1/2b and 1/2c were also present. The presence of these disease causing serotypes on raw chicken carcasses indicates that there is a risk for cross-contamination and subsequent infection.

The high mortality rate of this pathogen has prompted the U.S. to set a zero tolerance level in RTE foods. RTE foods are of greater concern because *Listeria* is a psychrotroph and has the ability to survive at 4°C. Although much of the concern for *Listeria* focuses on cooked and fermented products related to RTE

seafoods and meats, recently there has been more attention given to incidences of *Listeria* occurring in raw poultry and other poultry products. In addition, there have been reported cases of infection associated with persons involved in poultry processing and handling [73].

Food survey studies conducted in other countries and in the U.S. also show a prevalence in conventional chickens, although no data are available on organic chickens. This study showed that 26 of 61 (42.6%) conventional and 48 out of 210 (22.9%) organic chickens had positive isolates for a total of 95 and 153 isolates respectively (Table 7). Although there are no data available for comparison with organic chickens presently other studies have shown that *L. monocytogenes* prevalence rates ranged from 10% to as high as 64% [1, 3, 4, 16, 27, 74, 81] in conventional chickens.

Although there are data available in the literature for comparison of conventional chickens, it is practical to conduct a conventional raw chicken prevalence survey within the Metro Washington DC area. This allows for better control over your samples and is more representative of the organisms in the present environment for both organic and conventional chickens.

L. monocytogenes isolated from foodborne outbreaks show that three serotypes are present greater than 95% of the time. They are serotypes 1/2a, 1/2b and 4b. Serotype 1/2a is the most frequently isolated serotype from food samples, while 4b is the most frequently implicated in foodborne outbreaks. However, this study found that in organic chickens only eight (16.7%) samples were of the 1/2a

serotype, while 4b was the dominant serotype with 36 (75.0%) isolates. This is significant for disease potential with risks factors included in improper food handling, since serotype 4b is most commonly implicated in food-borne outbreaks.

Although serotyping is generally done using antisera, this study used a recent PCR method to determine if the isolates from the samples belong to the most common serotypes that cause disease. The PCR primers, which targeted specific regions in each of the target serotypes were designed based on lineage specificity. All three of the lineages were included in the serotyping analysis and were distinguished based on negative and positive results from the PCR reactions.

Both antisera and PCR serotyping methods have advantages and disadvantages. Although agglutination serotyping methods are costly, time consuming and require highly trained skills, they are important because they use antiserum to detect serological 'H' and 'O' antigens, which is the principle behind serotyping. However the PCR method used, was developed by a group of researchers [9], who not only performed PCR serotyping but used the traditional agglutination serotyping on the same isolates. The results indicated that there was a greater than 90% agreement of isolate serotypes for all samples, between these two methods. This suggests that the PCR serotyping primers are reliable and may have the potential to become a more acceptable standard method in the near future.

Following serotyping, PFGE can be performed to determine the genetic relatedness between the serotypes. The dendrogram generated show that there are

two distinct clusters, which separate lineages I and II, when comparing among the serotypes found on the isolates in this study. The first cluster contained those isolates which are from the 1/2a and the only 1/2c serotype, while the second cluster contain the 4b and 1/2b serotypes. This is not unexpected. Other studies have indicated that PFGE has been able to differentiate isolate serotypes among the major lineages [13, 60].

Using PFGE to subtype has been shown to be highly discriminatory as well. It is not only used for epidemiological tracking of clones implicated in food borne outbreaks and clinical isolates but to examine the genomic variation in samples. In this study it was able to differentiate 37 different genomic patterns out of the 74 isolates.

For the 1/2a serotype, all except four samples had a unique banding pattern. There were a total of 11 isolates with nine different patterns. This correlates with previous data which indicates that the serotype is genetically diverse with almost as much as twice the genetic variation than other serotypes [8, 9].

In a study using PFGE among several laboratories, the majority of epidemiologically related strains were subtyped and correctly identified. This study also determined that PFGE subtyping of serotype 4b is very useful and discriminatory. This subtype is historically difficult to discriminate by other subtyping methods because clones within the serotype can be so genetically similar. Other studies have also shown that 4b is not genetically diverse and more

homogenous than the other serotypes [7, 37, 51, 67] although there is evidence that there may be more than one lineage within the 4b serotype [90]. The differences within the 4b serotype has been speculated to play a role in the serotypes virulence potentials as well [97].

The 4b serotype patterns did not seem to be uniquely different from each other or from the 1/2b serotype. In fact, there were two 4b and 1/2b serotypes patterns which had identical banding patterns. This reinforces previous studies which indicate there may be an ancestral lineage between the 1/2b and 4b serotypes. It has been reported that the 4b serotype may have branched from the 1/2b serotype by gene acquisitions or gene losses to its genome [97].

Additionally the 4b and 1/2b serotypes seemed to cluster together and from the BioNumerics dendrogram are all related to the same lineage and cluster. There has also been evidence that suggests that there is a common origin of the serotypes 1/2b, 3b and 4b, [90], although more work needs to be done to clarify the exact origin and evolution of these serotypes.

There was not a distinct pattern when the patterns for the conventional and organic chickens were compared. This may be due to the fact that there were not enough conventional chickens to do an adequate comparison.

Conclusion

Despite having made important scientific advances in understanding the genus *Listeria* and its six species, much is still to be discovered of this pathogen especially on new and emerging markets such as in the organic foods market. Therefore an effort should be made to continue to perform surveillance research on the safety of animals and all foods produced under the organic seal. This study is one of the few which demonstrates that there indeed is a prevalence of *L. monocytogenes* on raw foods and one of the first to demonstrate a prevalence on organic foods. This in turn can be dangerous if improper food handling occurs either at the processing level and/or at the consumer level.

In addition, serotyping and subtyping isolates of the pathogen *L. monocytogenes* is not only important for epidemiological reasons but for increasing our knowledge about the ancestry, evolution and virulence of this important foodborne pathogen.

In order to better understand the relationship between the prevalence of *Listeria* on organic and conventional chickens, the experimental design of this study needs improvement. Although there was a sufficient amount of organic isolates, 281 over one year collection period, there were only 61 conventional isolates over a three month time frame. It would be necessary to continue the isolation from additional conventional chickens in order to get a better idea of how the prevalence rates relate in chickens raised in these two different environments.

Future work can also be done on the remaining isolates which were untested in this study. It would be interesting to understand the virulence potentials among these isolates, especially those included in the 4b serotype because they are most often implicated in outbreaks which cause disease. Since only one isolate from each of the positive *L. monocytogenes* chicken samples were tested, the remaining isolates can also be examined for their clonality as well as their serotype and genetic diversity. Lastly, an antimicrobial susceptibility profile would be of significant value. Because these isolates came from organic chickens, they should exhibit less resistance to antimicrobials commonly associated with *Listeria* antimicrobial resistance.

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