Project Title: Prevalence of *Listeria monocytogenes* in Beef Processing Plants

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Background

Contamination has been an issue plaguing the original PCR experiments. After ordering new solutions and still seeing contamination, we concluded that the problem did not lie with the reagents, the equipment or the graduate students techniques. The problem must have thus been associated with the oligo-primer set developed. There must have been a problem with nonspecific binding of the primers allowing for 75 bp sections of other DNA sequences to be amplified. Also, it was possible that the materials received from the vendors weren't as specific as we wanted. The primers could have had some additional oligo contained in them, since theoretically one extra sequence present could yield 2n times more products, more than enough to pose PCR contamination problems.

The final target of these studies has always been to develop a highly sensitive assay for the trace detection of prion proteins in body fluids. iPCR, which combines the specificity of immunoassays and the amplification power of PCR, is an ultrasensitive technique for trace analysis of proteins and antigens. iPCR usually lowers the LOD for protein detection about three (3) orders of magnitude, when compared to conventional ELISA. This approach requires: (1) immobilizing a prion protein to a solid support surface; (2) linking thru the detector Ab with a DNA label; (3) amplifying the DNA label by PCR; and (4) subsequently detecting amplification products. The procedure has been been illustrated in Figure 1 (and variations thereof).

One critical aspect of iPCR is the efficient coupling of the target-specific Ab (detector) with the oligo (to form the immunocomplex or chimera). Typically, this was achieved by successive coupling steps of several components. This strategy needs a large number of incubation steps, and usually entails an incomplete interphase coupling of the reagents, occurring with an efficiency of only ~10% for each step. In order to circumvent this problem, we tried to use synthetic strategies based on self-assembly of molecular building blocks to get the immunocomplex (Ab-oligo chimera) for iPCR. This method seemed to be an effective approach to couple several components for iPCR. Its advantage includes high sensitivity, high reproducibility, high linearity, time savings, single step protocol, minimization of signal loss due to inefficient coupling during incubation, and an improved ease of handling. We are not the first to consider using a chimera, but it requires the in-house synthesis, purification, isolation, and characterization before it can be routinely applied in a iPCR format.

Another common problem in immunoassays is nonspecific binding. We performed a series of comparison studies to understand where this came from, and found it mainly occurred between the capture Ab and detector Ab in the immunocomplex formation step. In order to decrease this unwanted binding, we took several steps, such as changing the concentration of capture and detector Ab, trying different blocking buffers and different wash procedures, and so forth. Further studies are in progress.

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The stated objectives for this work were: To assess the prevalence, levels, genetic fingerprints and antibiotic susceptibility of *L. monocytogenes* isolated from the beef-processing environment.

Methodology

***Sample collection and culturing for* L. monocytogenes.** Five hundred environmental and animal-associated (hide and carcass) samples were collected monthly from each of two beef processing plants over the course of five months between April and October 2002 for a total of 5,000 samples. These samples were analyzed for the presence or absence of the pathogen *L. monocytogenes* by two commercial laboratories. Multiple isolates from each positive sample (one confirmed *L. monocytogenes* isolate, isolate 'a' and 3 presumptive *Listeria* isolates, isolates 'b', 'c' and 'd') were then sent to ARS for further analyses.

Storage of isolates. Upon receipt, each isolate was streaked for isolation onto a brain heart infusion agar plate (BHI) and incubated overnight at 37°C. Following incubation, plates were examined for purity and individual isolates were stored at –20°C in 1-ml aliquots of BHI broth with 10% glycerol.

Confirmation of presumptive* L. monocytogenes *isolates. Each isolate (a, b, c and d from each positive sample) was streaked onto ALOA agar, a selective and differential medium for the isolation of Listeria spp. and for the presumptive identification of L. monocytogenes. *L. monocytogenes* are differentiated from other *Listeria* spp. by the presence of an opaque halo around a light blue colony. These presumptive *L. monocytogenes* isolates were then further characterized by biochemical testing using the API-Listeria (Analytical Profile Index) test strips, according to the instructions of the manufacturer. All *L. monocytogenes* that were ALOA-positive and positive on *Listeria* API strips were then subtyped by pulsed field gel electrophoresis.

Molecular subtyping via pulsed-field gel electrophoresis. From each *L. monocytogenes*-positive sample, up to four isolates (a, b, c and d) were subtyped by the pulsed-field gel electrophoresis (PFGE) technique of contour-clamped homogeneous electric fields (CHEF) electrophoresis, as previously described by Harsono et al. (1993). Briefly, genomic DNA was digested in agarose plugs with SmaI (Promega Corp., Madison, WI), as recommended by the manufacturer. The resulting DNA fragments were resolved by CHEF-PFGE using a CHEF-DRII apparatus (Bio-Rad Laboratories, Richmond, CA) at 200 V for 22 h at 14°C and switch times from 1 to 60 s. Low-range lambda concatamers (Promega) were used as DNA size standards. The fragments were visualized using ethidium bromide (10 µg/ml; Bio-Rad) and short wave ultraviolet light. Genomic profiles generated using SmaI and PFGE were analyzed and compared using the Gel Doc 1000 system (BioRad). The presence and absence of restriction fragments were ascertained by the system software using fragments 50-Kb or longer in length. The similarity of the profiles was determined visually. In general, multiple isolates (a, b, c and d) from a positive sample had the same PFGE profile, so the profile from only one isolate, normally the 'a'/confirmed isolate was used as the representative PFGE profile for comparison purposes. In cases where multiple isolates from one positive sample had different PFGE profiles, all different PFGE profiles were used for comparison. According to the methodology of Tenover et al. (1997), isolates with the same band pattern were said to be indistinguishable, isolates with a 2-3 band among 20 total fragments were said to be highly related and profiles with a greater than 3 band difference were said to be unrelated *L. monocytogenes* isolates.

Ribotyping. From each *L. monocytogenes*-positive sample, up to four isolates (a, b, c and d) were subtyped using the automated RiboprinterR (Qualicon/Dupont, Wilmington, DE),

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microbial characterization system and the restriction endonuclease EcoRI, as specified by the manufacturer.

Antibiotic susceptibility testing. *L. monocytogenes* isolates from each representative PFGE profile were tested for susceptibility to 18 antimicrobials using a custom-made panel on a semi-automatic broth microdilution system (Sensititre™ Trek Diagnostics, Westlake, OH), as per manufacturer's instructions. The 18 antimicrobials used were amikacin, amoxicillin/clavulanic acid, ampicillin, apramycin, cefoxitin, ceftiofur, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, imipenem, kanamycin, nalidixic acid, streptomycin, sulfamethoxazole, tetracycline, trimethoprim/sulfamethoxazole. National Committee for Clinical Laboratory Standard Guidelines were followed and the antimicrobials were used in concentrations as previously described by the National Antimicrobial Resistance Monitoring System (NARMS; http://www.arru.saa.ars.usda.gov) with the exception of imipenem, which was used at 0.25 to 8.0 µg with breakpoints of <4 for sensitive and >16 for resistant.

Findings

Prevalence results. In total 526 *L. monocytogenes* isolates, representing 141 *L. monocytogenes*-positive samples were sent to the ARS lab by the two commercial laboratories. Confirmatory testing of the isolates from each positive sample by the ARS lab (ALOA plates and the use of API Listeria) revealed that some of the isolates, including some "positive" 'a' isolates, were not *L. monocytogenes*. These isolates were found to be *L. innocua*, *L. ivanovii* or *L. welshimeri* and were not included in the study. The overall prevalence of *L. monocytogenes* in both plants over the sampling period was found to be 3% (141 positive samples from a total of 5000). A breakdown of monthly and plant prevalence can be seen in Table 1. Total monthly prevalence (plant 1 + plant 2) varied from 1% in August to 6% in May. There was a marked difference between the prevalence of the pathogen in plant 1 and plant 2, with 96% of the *L. monocytogenes* isolates recovered from plant 2. Indeed, the pathogen was not recovered from plant 1 during the sampling months of July, August and October, while in plant 2 it was recovered at a rate of between 2-5% for these months. The mean prevalence of the pathogen from plant 1 was 0.2% (0% - 0.6%), while the mean prevalence in plant 2 was 5.4% (2% - 11%).

Environmental and Animal-Associated Niches. Environmental and animal-associated niches for *L. monocytogenes* within the two processing plants were identified and a breakdown of these can be seen in Table 2. The pathogen was predominantly isolated from pens (8%) and hides (9%), but also from product contact surfaces, e.g. belts, (4%), slaughter floor drains (0.3%) and from beef carcasses (0.3%). Comparing plant 1 and plant 2, there was marked difference in the prevalence of *L. monocytogenes* in the environmental and animal-associated niches identified. In plant 2, 18% of the cattle hides and 15% of the cattle pens examined were positive for the pathogen, resulting in contamination of 8% of the product contact surfaces examined on the fabrication floor and 0.6% of the carcasses. In contrast, in plant 1, *L. monocytogenes* was only found on 0.6% of the hides and 1.3% of the pens examined, at the rate of 0.3% on the fabrication floor and was not detected on any of the carcasses examined.

PFGE analyses. Fifty-nine distinct PFGE profiles were identified from the 526 *L. monocytogenes* isolates, representing 141 positive samples. Table 3a and 3b present summaries of the PFGE analyses of the *L. monocytogenes* isolates. Appendix 1 gives a detailed account of the isolates in each PFGE profile in Table 3a. Of these 59 PFGE profiles, representing 141 positive samples, 35 were distinct profiles, being only found in one positive sample, while 24

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of the PFGE profiles represented 106 positive samples, with 2-16 positive samples per profile. Three PFGE profiles, # 1, 7 and 14 were the most commonly occurring profiles, accounting for 44 (31%) of the 141 positive samples studied. These profiles were found predominantly on animal hides (57%), but were also spread to product contact surfaces (39%) and subsequently to the bovine carcass (5%). In general, multiple isolates from the same positive sample had the same PFGE profile. However, in some instances two to three distinct profiles were observed from one positive sample. For example, sample number 634, a hide isolate recovered from plant 2 in April, displayed two distinct PFGE profiles (# 32 and 33), indicating two different, unrelated isolates of *L. monocytogenes* were recovered from this sample. Several PFGE profiles were found in all three sampling times of the month and across several months of sampling. For example, PFGE profile # 1 was recovered in separate sampling periods in April and was also recovered in May, July and August. In other instances, a PFGE profile was only isolated in one particular month. Profile # 14 is such an example. In this instance, 15 positive samples (11% of total samples) recovered from hides in plant 2, revealed the same PFGE profile, but this profile was only recovered from one sampling period in the month of May and was not recovered thereafter. In general the same PFGE profiles of *L. monocytogenes* were not found in both plant 1 and plant 2. However, there is one notable exception to this, where profile # 2 and 3 were recovered from pens and hides in plant 1 (positive samples # 449 and 450) and plant 2 (positive samples, 249, 250, 630, 647, 1038, 1052 and 1171) during the month of April.

Ribotyping analyses. Ribotyping analyses on all the April *L. monocytogenes* isolates identified 8 different ribogroups (results not shown). However, PFGE analyses of the same isolates revealed 16 different profiles. Based on these results it was decided that PFGE analyses was a more discriminatory subtyping method and hence ribotyping analyses was not performed on subsequent isolates.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing of the isolates was not performed. Such experiments can be conducted and completed within the next 6 months through our ongoing collaboration with Dr. Paula Cray.

Implications

In the present study, the prevalence of *L. monocytogenes* in the 5000 environmental and animal-associated samples collected monthly for five months was found to be relatively low at 3%. The present study also demonstrated that there was a marked difference between the prevalence of the pathogen among the processing plants, with 96% of the *L. monocytogenes* isolates recovered from plant 2. Recovery of the pathogen was also greater in April (3%) and May (6%) than in the subsequent months of July (2%), August (1%) and October (2%). Environmental and animal-associated (hide and carcass) niches for L. monocytogenes within the two slaughter plants were identified in the present study. The pathogen was predominantly isolated from pens (8%) and hides (9%). Hide contamination with the pathogen subsequently resulted in the contamination of product contact surfaces, e.g. belts, (4%), slaughter floor drains (0.3%) and ultimately entry into the food chain via contamination of bovine carcasses. This carcass contamination was low at 0.3%, indicating that current hygiene practices and intervention strategies used in these plants are preventing the vast majority of carcass contamination. However, this study has established that the bovine hide is a significant source of L. monocytogenes and further interventions focusing on the decontamination of the bovine hide may help decrease the rate of carcass contamination with this pathogen. The present study identified 59 distinct PFGE profiles from 526 *L. monocytogenes* isolates. Of these 59 profiles, 55

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were isolated in one sampling month and never thereafter. *L. monocytogenes* is commonly associated with cattle, who during lairage and slaughter introduce different strains of the pathogen into the processing plant, accounting for its heterogeneous nature as demonstrated in the present study. Four of the 59 PFGE profiles in the present study were detected across several months of sampling. Profile 1, is one such example, being isolated in May, July and August. This demonstrates the longevity of the organism and its' ability to survive over long periods and contaminate the processing environment. In general, multiple isolates from the same positive sample had the same PFGE profile. However, in some instances two to three distinct profiles were observed from one positive sample, demonstrating that more than one strain of *L. monocytogenes* was present in the sample. In general the same PFGE profiles of *L. monocytogenes* were not found in both plant 1 and plant 2. However, there were two exceptions to this, where profiles # 2 and 3, was recovered from hides and pens in plant 1 and plant 2 during the month of April.

Table 1: Monthly prevalence of *Listeria monocytogenes* in two slaughter plants

Month Sampled	Total Prevalence (Plant 1 + Plant 2)	Plant 1 only	Plant 2 only
April	3% (31/1000)*	0.6% (3/500)	6% (28/500)
May	6% (59/1000)	0.4% (2/500)	11% (57/500)
July	2% (17/1000)	0% (0/500)	3% (17/500)
August	1% (11/1000)	0% (0/500)	2% (11/500)
October	2% (23/1000)	0% (0/500)	5% (23/500)

* Five hundred samples were analyzed for the presence of *L. monocytogenes* monthly from each of the two slaughter plants, plant 1 and plant 2, to give a total of 1000 samples monthly

Table 2: Prevalence of *Listeria monocytogenes* in environmental and animal-associated niches in the slaughter plants

Sample type	Location of positive samples in plants	Total prevalence (plant 1+ plant 2)	Prevalence in plant 1	Prevalence in plant 2
	Pens	8% (12/150)	1.3% (1/75)	15% (11/75)
	Saws	0.4% (1/240)	0% (0/120)	0.8% (1/120)
Environmental	Product contact surfaces	4% (24/600)	0.3% (1/300)	8% (23/300)
	Slaughter floor drains	0.3% (1/360)	0% (0/180)	0.6% (1/180)
	Miscellaneous	0% (0/500)	0% (0/250)	0% (0/250)
Animal-related	Hides	9% (97/1050)	0.6% (3/525)	18% (94/525)
	Carcasses	0.3% (6/2100)	0% (0/1050)	0.6% (6/1050)

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Table 3a: PFGE profiles of *L. monocytogenes* isolated from two slaughter plants over a seven-month sampling period

Profile Number	Number of isolates in profile*(% of positive samples)	Months isolated	Plant isolated from	Niches isolated from
1	16 (11%)	May, July and August	2	Hides and product contact surfaces
2	7 (5%)	April	1+2	Hides and pens
3	3 (2%)	April	1+2	Hides
4	2 (1%)	April	2	Pens
5	2 (1%)	April	2	Pens and product contact surfaces
6	7 (5%)	May	2	Hides
7	13 (9%)	October	2	Hides and product contact surfaces
8	3 (2%)	April	2	Pen panels
9	2 (1%)	May and August	2	Hides
10	3 (2%)	April	2	Pens, hides and carcasses
11	7 (5%)	April and May	2	Pens, hides and carcasses
12	7 (5%)	May and October	2	Hides, product contact surfaces and carcasses
13	4 (3%)	May	2	Hides and carcasses
14	15 (11%)	May	2	Hides
15	3 (2%)	May	2	Hides
16	5 (4%)	July	2	Pens, hides and carcasses
17	5 (4%)	May	2	Pens and hides
18	3 (2%)	May	2	Hides
19	2 (1%)	August	2	Product contact surfaces
20	2 (1%)	October	2	Hides
21	2 (1%)	October	2	Product contact surfaces
22	3 (2%)	October	2	Hides
23	2 (1%)	October	2	Hides
24	3 (2%)	October	2	Hides

* See appendix 1 for isolate numbers in each PFGE profile

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Table 3b: PFGE profiles of *L. monocytogenes* isolated from two slaughter plants over a seven-month sampling period

Profile Number	Number of isolates in profile (isolate #)	Months isolated	Plant isolated from	Niches isolated from
25	1 (231a) ϕ	April	2	Hide
26	1 (247a)	April	2	Hide
27	1 (251a)	April	2	Hide
28	1 (370a)	April	2	Pens
29	1 (371a)	April	2	Pens
30	1 (569a)	April	1	Pens
31	1 (612a)	April	2	Product contact surface (saw)
32	1 (634a)	April	2	Hide
33	1 (634b)	April	2	Hide
34	1 (647b)	April	2	Hide
35	1 (1033a)	April	2	Hide
36	1 (1035a)	April	2	Hide
37	1 (1139a)	April	2	Floor drain
38	1 (1430b)	May	2	Hide
39	1 (1435a)	May	2	Hide
40	1 (1437a)	May	2	Hide
41	1 (1448a)	May	2	Hide
42	1 (1449c)	May	2	Hide
43	1 (1454a)	May	2	Hide
44	1 (1628a)	May	1	Hide
45	1 (1832a)	May	2	Hide
46	1 (1846a)	May	2	Hide
47	1 (1850a)	May	2	Hide
48	1 (2017a)	May	1	Product contact surface (belt)
49	1 (2232a)	May	2	Hide
50	1 (2238a)	May	2	Hide
51	1 (2242a)	May	2	Hide
52	1 (2258a)	May	2	Hide
53	1 (3041a)	July	2	Hide
54	1 (4225a)	August	2	Hide
55	1(4248a)	August	2	Hide
56	1 (4250a)	August	2	Hide
57	1 (4356a)	August	2	Product contact surface (belt)
58	1 (5831b)	October	2	Hide
59	1 (5959a)	October	2	Product contact surface (belt)

ϕ where a, b, c, or d is one of the multiple isolates from each positive sample sent to ARS for analysis. In most cases all four isolates had the same PFGE profile, so only one isolate profile, normally from isolate 'a' was used as the representative PFGE profile for comparison purposes. In cases where multiple isolates from one positive sample had different PFGE profiles, all different PFGE profiles were used.

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Appendix 1

Isolates numbers for PFGE profiles 1-16 in Table 3a

PFGE Profile*		
Main profile	Subprofile	Isolate numbers
	A	1437b, 1447a
	B	2352a, 2356a
1	C	2632a, 2636a, 2652a, 2659b, 2673b, 2677c, 2750a, 2751a, 2756a, 2757a, 3156a, 3157a
	D	3953a, 4355a, 4357a, 4751a
	A	249a, 449a
	B	1038a, 1171a
2	C	630a
	D	647a
	E	655a
3	A	250a. 450a, 1052a
4	A	1170d
	B	1172b
5	A	1019a
	B	1170a
	A	1456a, 1840a, 1847a, 1859a
6	B	1436a
	C	2237a
	D	2254a
7	A	5018d, 5048a, 5151a, 5551b, 5819a, 5822a, 5842a, 5857a, 5844a,, 5953a, 5955a, 5958a, 5959b
8	A	369a, 1041a, 1077a,
9	A	1825a
	B	4256a
10	A	369a, 1041a, 1077a
	A	1169a, 1171d, 1836a, 1873a
	B	635a
11	C	248a
	D	1170c
12	A	1826a, 1827a, 1865a, 5843a, 5844c, 5849d, 5854c
13	A	1441a, 1445a, 1837a, 1872a
14	A	1828a, 1831a, 1833a, 1834a, 1839a, 1841a, 1842a, 1843a, 1844a, 1849a, 1851a, 1852a, 1853a, 1854a, 1856a
15	A	1449a, 1451a, 1835a
16	A	2659a, 2673a, 2769a, 3028a, 3039a

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Appendix 1 continued:

Isolates numbers for PFGE profiles 1-16 in Table 3a

17	A	1838a, 1845a, 1855a, 1969a
	B	1858a
18	A	1430a, 1434a
	B	1452a
19	A	4351a, 4352a
20	A	5857d, 5953d
21	A	5018a, 5551a
22	A	5046a
	B	5831a
	C	5843a
23	A	5830a, 5839a
24	A	5052a, 5055a,
	B	5832b

* Isolates within a PFGE profile can be divided into a main profile and a subprofile. Isolates within a subprofile are indistinguishable, while isolates within a main profile are highly related and show less than a 4-band difference among 20 total fragments.

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