Journal of Food Protection, Vol. 69, No. 4, 2006, Pages 815–819

Prevalence and Typing of *Listeria monocytogenes* in Raw Catfish Fillets†

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MS 05-406: Received 31 July 2005/Accepted 6 November 2005

ABSTRACT

Raw channel catfish fillets collected from three processing plants during four time periods were tested for the presence of *Listeria* species. *Listeria monocytogenes* was the predominant *Listeria* species found in these catfish fillets, with 25 to 47% prevalence. Other *Listeria* species, such as *L. welshimeri, L. innocua, L. ivanovii, L. grayi,* and *L. seeligeri,* were also found. *L. monocytogenes* isolates were further fingerprinted by a repetitive element PCR. Forty distinctive electrophoretic types (ETs) and three genetic clusters were determined by Dice coefficient analysis and UPGMA (unweighted pair group method using arithmetic averages). Twenty of 40 ETs were represented by a single isolate, and the other 20 ETs were represented by 2 to 11 isolates. Thirty-five ETs, represented by 76 isolates, were found in processing plant A, B, or C and designated plant-specific types. The remaining five ETs, represented by 21 isolates, were found in multiple plants and designated nonplant-specific types. In addition, 10 ETs from 52 isolates were found repeatedly during different seasons. Plant-specific and nonplant-specific *L. monocytogenes* coexisted in processed catfish fillets. Some isolates were persistently found in processed fillets, suggesting that either the current sanitation procedures used by these plants are inadequate or that these isolates originated from the natural habitats of the catfish. The results also suggest that the repetitive element PCR is a useful tool for differentiating *L. monocytogenes* subtypes and can be used for tracing the source of a contamination.

Listeria monocytogenes has been recognized as an important foodborne pathogen in humans, especially for high-risk groups such as pregnant women and immunocompromised individuals *(18, 23).* Several studies have reported that seafood products, including ready-to-eat (RTE) products such as smoked salmon, shrimp, rainbow trout, crawfish, and fish salads, are also contaminated with *L. monocytogenes (1, 5, 9, 12, 25)*; however, little is known about the prevalence of *L. monocytogenes* contamination in catfish products. *L. monocytogenes* is ubiquitous and has been found in raw catfish *(29)* as well as in other raw meat products such as chicken, beef, and pork *(17, 24).* Because of the ability of *L. monocytogenes* to grow at refrigeration temperatures, the presence of this bacterium, even in low numbers, may pose a risk to human health when fish products have been stored at refrigeration temperatures for long time periods. Although, to our knowledge, no human listeriosis case has been reported due to the consumption of catfish products, a potential risk may still exist if cross-contamination occurs between raw products and RTE products.

Repetitive element PCR (rep-PCR) is currently recognized as one of the most popular characterization methods for fingerprinting microorganisms in food and water *(15, 20, 27).* In rep-PCR DNA fingerprinting, oligonucleotide primers based on short repetitive sequence palindromes (REP) elements are used to amplify the genomic DNA between adjacent repetitive extragenic elements to obtain strain-specific DNA fingerprints that can be analyzed with a band pattern recognition computer software *(6, 13).* rep-PCR has been successfully used for typing and differentiating *L. monocytogenes* isolates from human clinical cases and food products *(13, 27). L. monocytogenes* is commonly found in soil and water and is considered an environmental organism. To prevent the potential contamination or cross-contamination of RTE products, it is as important to monitor *L. monocytogenes* in food-processing environments as in end product testings. However, to our knowledge, no information is available about the prevalence of *L. monocytogenes* or the subtypes of this organism found in catfish processing plants. The objectives of this study were to (i) determine the prevalence of *L. monocytogenes* and (ii) understand the distribution of subtypes of *L. monocytogenes* in the processing plants.

MATERIALS AND METHODS

Sampling. A total of 240 channel catfish fillets were collected from three processing plants (A, B, and C) at four time periods (summer, fall, winter, and spring). The three plants were at least 50 mi (80 km) apart, and all three plants were unrelated, operating independently. Plants A and B were large commercial operations, and plant C was a small, family-owned operation. Twenty skinless and boneless fillets were collected from each plant per time period (3 plants × 4 times). Each fillet weighed approximately 200 to 250 g. The first fillet was collected 1 h after the beginning of processing at the end of the processing line, and the rest of the fillets were collected at 20-min intervals. Each fillet

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† Contribution J-10776 from the Mississippi Agriculture and Forestry Experiment Station.

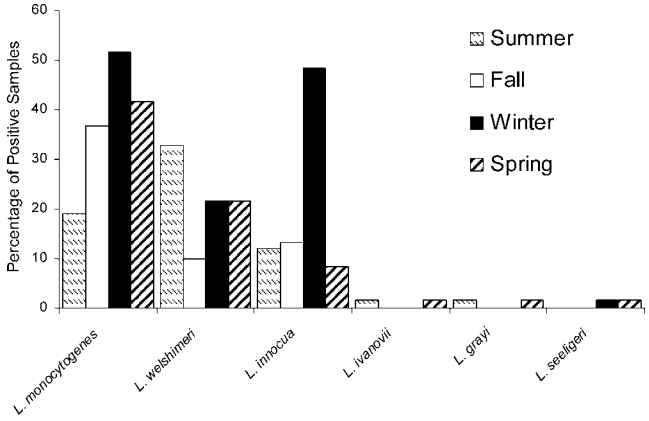

FIGURE 1. *Distribution and prevalence of* Listeria *spp. isolated from channel catfish fillets for four seasonal time periods. Results are the compilation for all three plants.*

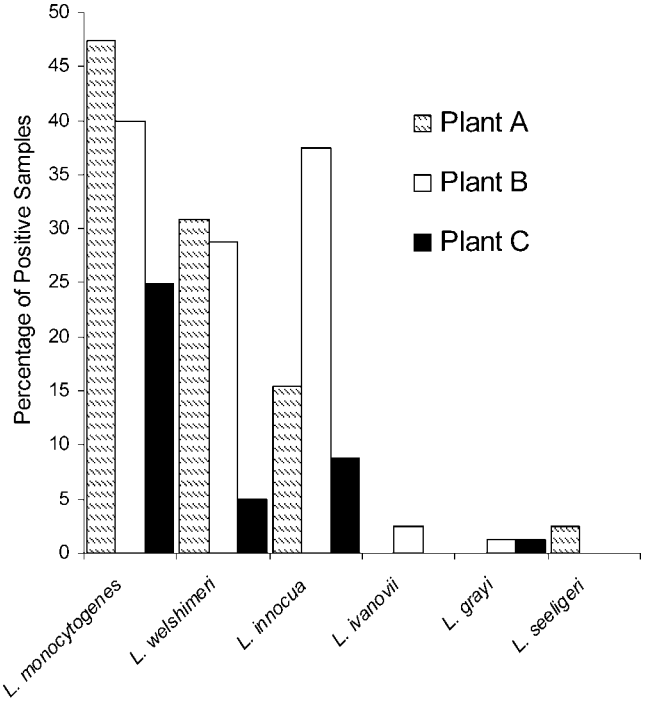

FIGURE 2. *Distribution and prevalence of* Listeria *spp. isolated from channel catfish fillets in three processing plants.*

was placed in a bag, and all samples were packed on ice and shipped to our laboratory for bacteriological analyses.

Bacterial identification. The isolation of *L. monocytogenes* followed the U.S. Food and Drug Administration procedure for seafood *(10).* Briefly, 25 g of catfish fillet and 225 ml of enrichment broth were placed in a stomacher bag and mixed in a Seward Stomacher 400 (Seward Laboratory, London, UK) for 1 min and incubated for 48 h at 30°C. After incubation, 1 loop of the bacterial culture was streaked onto an Oxford agar plate and incubated at 35°C for 48 h. A black colony with a black halo that was due to the hydrolysis of esculin was picked from the plate. A total of three colonies were selected from each plate and confirmed by a commercial API *Listeria* kit (bioMérieux, Hazelwood, Mo.).

Fingerprinting of isolates by rep-PCR. Genomic DNA of *L. monocytogenes* from each positive fillet sample was extracted with a DNeasy Tissue Kit (Qiagen, Valencia, Calif.). Extracted DNA was dissolved in 80 μl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and the concentration of DNA was measured by spectrophotometry at 260 nm. Bacterial genomic DNA (25 ng) was subjected to PCR with the primers REP 1R-I (5′-III ICG ICG ICA TCI GGC-3′) and REP 2-I (5′-ICG ICT TAT CIG GCC TAC-3′) *(27).* The nucleotide inosine (I) was included in the primers at ambiguous positions in the REP consensus *(13, 27).* The amplification reactions were performed in the final volume of a 25-μl solution containing 25 pmol of each of the primers, 250 μM of each deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 10× PCR buffer, 2% dimethyl sulfoxide, and 1.0 U of JumpStart *Taq* DNA polymerase. The deoxynucleoside triphosphates were purchased from Applied Biosystems (Foster City, Calif.), and the other PCR reagents were purchased from Sigma (Woodlands, Tex.). Amplifications were performed in a DNA thermocycler (GeneAmp PCR System 9700, Applied Biosystems) with the following temperature profile. After initially being heated to 95°C for 2 min, the mixture was subjected to 35 cycles of amplification. This consisted of denaturing at 94°C for 3 s and then at 92°C for 30 s, annealing at 50°C for 60 s, and then extension at 65°C for 8 min. This process was followed by a final extension at 65°C for 8 min.

The rep-PCR products (7.5 μl) mixed with 2.0 μl of 10× gel loading buffer (Invitrogen, Carlsbad, Calif.) were separated on a 1.5% agarose gel. A 1-kb plus DNA ladder (Invitrogen) was also loaded into every sixth well of the gel. The 25-cm-long gels were electrophoresed in 0.5× Tris-borate-EDTA buffer (Amresco, Solon, Ohio) at 95 V for 10 h. After staining with GelStar nucleic acid gel stain (BMA, Rockland, Maine), the gel images were captured under UV transillumination with an AlphaImager Image System (Alpha Innotech Co., San Leandro, Calif.). The images were digitized and transferred to Bionumerics 3.0 software (version 3.0, Applied Maths, Kortrijk, Belgium) for pattern analysis. The normalization was adjusted with the 1-kb plus ladder (Invitogen). The gels were then carefully verified by visual examination to correct for unsatisfactory detection *(13).* Grouping was performed with the band position tolerance of the Dice coefficient at 1.0%, and cluster analysis for genetic similarity was performed by UPGMA (unweighted pair group method using arithmetic averages) protocols.

RESULTS

Of the 240 fillets examined, 37.4, 21.4, 20.6, 0.8, 0.8, and 0.8% were positive for *L. monocytogenes*, *L. welshimeri*, *L. innocua*, *L. ivanovii*, *L. grayi*, and *L. seeligeri*, respectively. *L. monocytogenes* was more frequently isolated from channel catfish processed in the winter (51.0%) when compared to samples processed in the spring (41.0%), fall (36.7%), and summer (19.0%) (Fig. 1). The prevalence of *L. monocytogenes* was 47.4% for plant A, 40.0% for plant B, and 25.0% for plant C (Fig. 2).

Various DNA patterns of *L. monocytogenes* isolated from raw processed catfish fillets showed multiple DNA fragments that ranged from approximately 350 to 5,000 bp with various intensities (Fig. 3). Ninety-seven DNA fingerprints from the isolates were classified into 40 distinctive electrophoretic types (ETs) by a band-based analysis. Twenty ETs were represented by a single isolate ($n = 20$ isolates), and the other 20 ETs were represented by 2 to 11 isolates ($n = 77$ isolates). Thirty-five ETs, represented by 76 (78.4%) isolates, were found in an individual plant and were recognized as plant-specific ETs (plant A: 13 ETs, 22

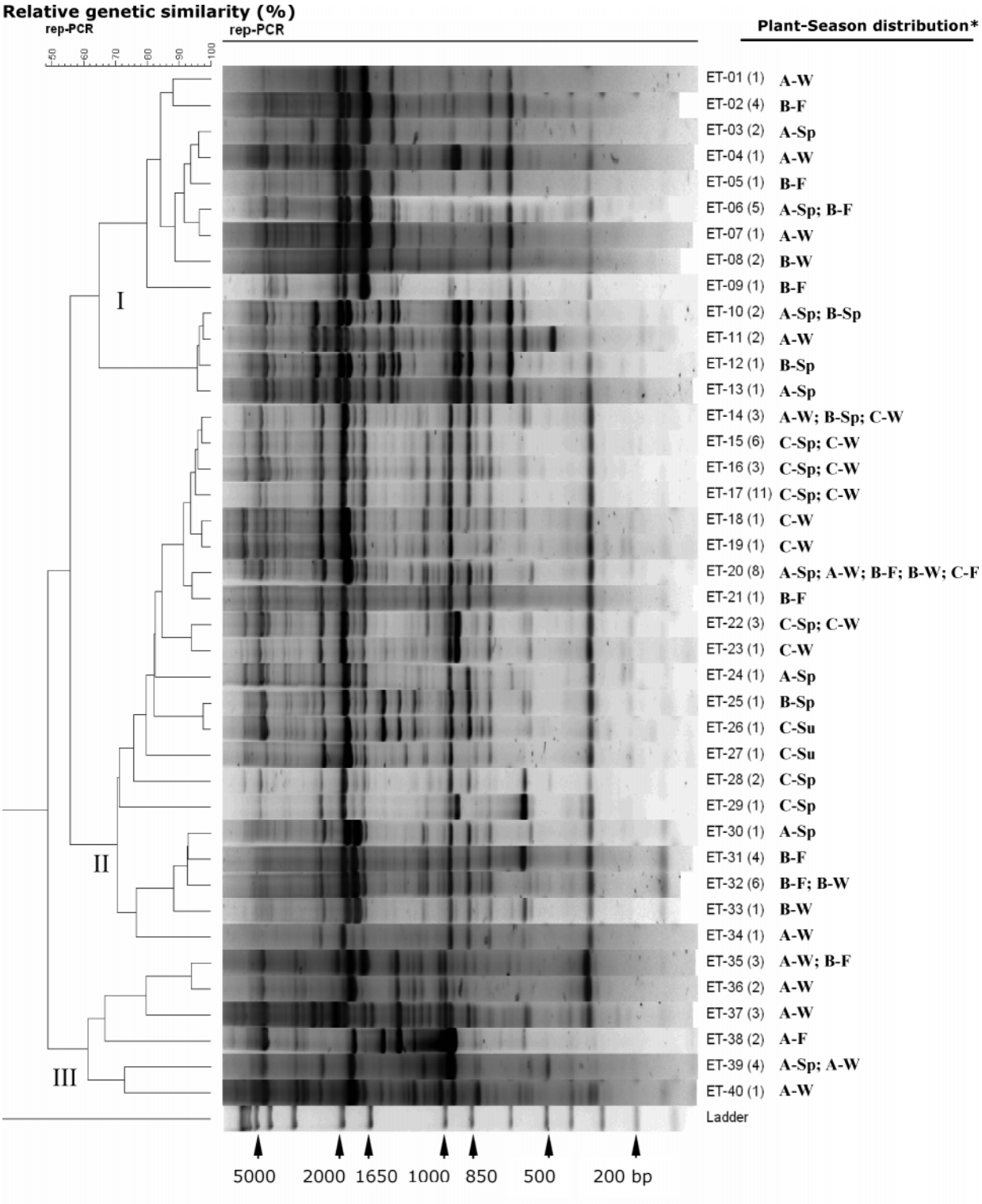

FIGURE 3. *Dendrogram of rep-PCR fingerprints for 40 ETs of* L. monocytogenes *isolates. The percentage of similarity is shown above the dendrogram. The isolate numbers belonging to each individual ET are shown in brackets. Cluster analysis and similarity values between fingerprints were based on the Dice coefficient. Band images as shown are software reconstructed and thus underestimate the clarity of the actual gel images. * Plant-season distribution: A, plant A; B, plant B; C, plant C; Sp, spring; Su, summer; F, fall; W, winter.*

isolates; plant B: 11 ETs, 23 isolates; and plant C: 11 ETs, 31 isolates). The other five ETs, represented by 21 (21.6%) isolates, were found in either two or all three plants and were designated nonplant-specific ETs. ET-17, represented by 11 isolates, was found in a single plant, and ET-20, represented by 8 isolates, was the most common ET and was found in multiple plants.

All isolates showing the same band pattern were considered an identical strain or the same ET *(26).* Therefore, one representative strain of a given ET was then selected,

and a similarity analysis was conducted with Dice coefficient and cluster analysis by UPGMA protocols. A total of 40 ETs were grouped into three genetic clusters (I to III) on the basis of a 55.0% relative genetic similarity (Fig. 3). Cluster II was the largest cluster, consisting of 21 ETs represented by 58 isolates from all of the three unrelated processing plants, whereas the other two clusters consisted of 6 and 13 ETs that were represented by 39 isolates. Eighteen ETs, represented by 32 isolates from plant A, and 15 ETs, represented by 32 isolates from plant B, were distributed in all three clusters. Thirteen ETs, represented by 33 isolates from plant C, were distributed only in cluster II. Overall, the isolates from plant C had the highest genetic similarity when compared to the isolates from plants A and B.

Ten ETs were found in samples taken during multiple seasons. Among these 10 ETs, 6 (ET-15, ET-16, ET-17, ET-22, ET-32, and ET-39) were found only in a single plant (ET-39 in plant A, ET-32 in plant B, and ET-15, ET-16, ET-17, and ET-22 in plant C); 2 (ET-6 and ET-35) were found in both plants A and B; and 2 (ET-14 and ET-20) were found in all three plants.

DISCUSSION

To our knowledge, this is the first report that describes the prevalence and subtypes of *Listeria* species in processed catfish. *L. monocytogenes* was the predominant *Listeria* species present in processed catfish. In our study, *L. monocytogenes* is more prevalent in the winter, which is probably because the bacterium can outcompete and exclude other bacterial species at low temperatures. Plant C had the lowest percentage of isolates, which may be because plant C is the smallest and processes fewer fish than the other two plants. However, the study was conducted during a 1-year period, and a comprehensive study is required to confirm whether the prevalence of *L. monocytogenes* in catfish correlates with seasonality. The prevalence of *L. monocytogenes* has been reported in soil and aquatic environments *(30)* as well as in meats, seafoods, dairy products, and vegetables *(1, 9, 17, 24, 25).* Because *Listeria* is widely distributed, the catfish-origin isolates detected in this study may have originated from the environment, e.g., catfish ponds, or the produce may have been contaminated in the processing plants.

Thirty-five (88%) of the 40 ETs were found in an individual plant, which suggests that even though *L. monocytogenes* is ubiquitous, the mechanism by which the subtypes were established in each individual plant was quite different. This may be because the catfish came from different sources or because the plants use different sanitation control procedures during processing. Several reports *(2, 4, 8, 28)* have also suggested that raw materials are an important initial contamination source in processing environments; however, others suggest that raw materials are not significantly related to the contamination of end products *(16).* Five ETs were found in multiple plants, and the processing procedures of these plants were unrelated. The five nonplant-specific ETs may be common flora in the environments in which the catfish were raised, or they may be highly resistant to disinfectants; therefore, these ETs persistently reside in processing environments.

In this study, isolates were recovered from samples collected during a 12-month period, and 10 ETs from 54% of the isolates were found repeatedly in the different seasons. Six ETs were found repeatedly in a single sampling plant. Our findings are in agreement with the findings of Rorvik et al. *(21, 22),* who found a certain strain that had persisted for 8 months in a smoked salmon product from the same producer. Our results suggest that persistently detected isolates and sporadically detected isolates can coexist in a single processing plant, as noted by Boerlin et al. *(3).* In the current study, different ETs were isolated simultaneously from a single fillet sample (data not shown), suggesting that co-contamination by different subtypes of *L. monocytogenes* is common. Multiseasonal distribution of isolates may be the result of persistent contamination sources or colonization within the plant environment over time. Once a plant is colonized with the particular subtypes, it is difficult to eliminate the contaminating subtypes *(5, 14, 19, 21).* Four ETs (ET-06, ET-14, ET-20, and ET-34) representing 20% of the isolates were found in different seasons and in unrelated processing plants, indicating that these subtypes are either ubiquitous or natural inhabitants of the catfish ponds or processing plants. Similar findings have also been described by other researchers *(8, 21, 31).*

Recent studies have reported the possible association between *L. monocytogenes* isolates from fish products and human listeriosis *(7, 11, 21),* although no cases have been reported that are related to the consumption of catfish products. However, catfish products contaminated with *L. monocytogenes* remain a potential risk to human health through cross-contamination during food preparation. For this reason, decreasing *L. monocytogenes* in raw catfish products is necessary to reduce the risk of cross-contamination and ensure the safety of catfish products. Fonnesbech Vogel et al. *(8)* reported that plants that conduct rigorous cleaning and disinfection have fewer DNA fingerprint patterns that are consistent with the persistence of *L. monocytogenes* subtypes. In our study, the three plants that were contaminated with the various ETs of *L. monocytogenes* and the presence of persistent ETs indicate that the sanitation procedures in the plants were inadequate. Therefore, improvements in hygiene and sanitation can be monitored through the tracking of ETs and persistently existing strains. With this information, plant managers can modify cleaning and sanitation procedures as a means of reducing and controlling microbial contamination.

ACKNOWLEDGMENTS

This work was supported by grants from the U.S. Department of Agriculture, Agricultural Research Service, no. 58-6202-5-083, and the College of Veterinary Medicine, Mississippi State University.

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