Turk J Vet Anim Sci
29 (2005) 1007-1011

Research Article

Isolation of *Listeria monocytogenes* from Fish Intestines and RAPD Analysis

Hasan Basri ERTAŞ*, Engin ŞEKER
Department of Microbiology, Faculty of Veterinary Medicine, Fırat University, 23119 Elazığ - TURKEY

Received: 22.03.2004

Abstract: One hundred and fifty fish caught from Keban Dam Lake were examined for the presence of *Listeria monocytogenes*. A random amplified polymorphic DNA (RAPD) assay using a random primer was performed to detect genetic variations among *L. monocytogenes* isolates. The selective enrichment procedure followed by selective isolation was used for listeria isolation from fish intestines.

Ten (6.6%) *L. monocytogenes* were isolated and identified from fish intestines. Two different band profiles were obtained by the RAPD analysis of the isolates.

The results indicated that the isolates might have originated from different sources, and fish contaminated with *L. monocytogenes* may cause serious health problems in humans.

Key Words: Fish, intestine, *Listeria monocytogenes*, RAPD

Balık Bağırsaklarından *Listeria monocytogenes* İzolasyonu ve RAPD Analizi

Özet: Keban baraj gölünden yakalanan toplam 150 balık, *Listeria monocytogenes* varlığı yönünden incelendi. *L. monocytogenes* izolatları arasındaki genetik farklılıkları tespit etmek amacıyla rastgele bir primer kullanan Random Amplified Polymorphic DNA yöntemi kullanıldı. Balık bağırsaklarından listeria izolasyonu amacıyla selektif zenginleştirme sonrası selektif izolasyon basamağı ile devam eden yöntem kullanıldı.

İncelenen balık bağırsaklarından 10 (% 6,6) *L. monocytogenes* izole ve identifiye edildi. İzolatların RAPD analizi sonrasında iki farklı bant profili elde edildi.

Bu çalışmanın sonuçları izolatların farklı kaynaklardan gelmiş olabilecegini ve balıkların insanlarda ciddi sağlık sorunlarına yol açabilecek olan *L. monocytogenes* ile kontamine olduğunu göstermektedir.

Anahtar Sözcükler: Balık, bağırsak, *Listeria monocytogenes*, RAPD

Introduction

Listeria spp. are considered ubiquitous organisms, widely distributed in the environment (1). *L. monocytogenes* has even been isolated from river water and sediment (2), canals and lakes (3). Listeriosis is an atypical foodborne disease that most frequently affects pregnant women, newborn infants, children and adults whose immune systems are weakened. The illness is rare but the mortality rate can be as high as 30%. Consumption of foods contaminated with *L. monocytogenes* is the primary route of transmission for Listeriosis (4).

Lennon et al. (5) proposed that consumption of shellfish and raw fish was responsible for an epidemic of prenatal listeriosis in New Zealand in 1980. Other researchers have reported sporadic cases of seafood-borne listeriosis (6,7).

A number of publications indicate that fish can frequently be contaminated with *L. monocytogenes* (8-12). The presence of Listeria species in fish has been reported by a number of researchers. The incidence rates vary between 35.7% and 0% in fresh fish according to Fuchs and Surendran (13), but were reported as 72.4%-7.2% by Jeyasekaran et al. (14).

* E-mail: hbertas@yahoo.com

The development of a rapid, accurate and discriminating typing method is needed to monitor listeriosis outbreaks. Of the several typing techniques developed to differentiate Listeria at subspecies level, serotyping was the most commonly and extensively used (15). However, the limited value of serotyping in Listeria typing was mentioned in several reports, stemming from the fact that only a minority of serovars were detected from field isolates (16).

Because of the low discriminatory power of this method, a phage-typing system was developed and was the only means to distinguish between strains of the same serovar before the introduction of molecular methods.

The drawback of this typing is that there are non-typable strains. Since 1989, various molecular typing methods have been applied to *L. monocytogenes*, including multilocus enzyme analysis, ribotyping, DNA microrestriction and macrorestriction profile analysis and random amplified polymorphic DNA (RAPD) assay (17-21).

In epidemiology, the RAPD assay is appropriate for screening large panels of strains. Many researchers have used this assay for typing *L. monocytogenes* isolates from different sources (20-22).

Numerous reports can be found on the presence of *L. monocytogenes* in fish and fish products (23,24). This study was performed to determine the presence of *L. monocytogenes* in fresh fish from Keban Dam Lake and to detect genetic variability in the isolates by RAPD analysis.

Materials and Methods

Sampling Procedure

One hundred and fifty fish (*Capoeta capoeta umbla*) were sampled from Keban Dam Lake and were analysed for the presence of *L. monocytogenes*. The fish were placed into separate plastic bags and immediately transferred to the Microbiology Laboratory at Firat University Faculty of Veterinary Medicine. Each fresh fish was opened with a sterile scalpel and about 1 g of intestine content was taken using a sterile swab for listeria isolation.

Isolation and Identification Procedure

Intestinal contents of the fresh fish were transferred to 10 ml Listeria Enrichment Broth (Oxoid). The tubes were shaken vigorously and incubated for 24-48 h at 37 ºC. One or two loops of Listeria Enrichment Broth culture were streaked onto Listeria Selective Agar (Oxoid). The plates were incubated for 48 h at 37 ºC. Plates were examined for typical Listeria colonies with dark halos and suspicious colonies were transferred onto tryptic soy agar (TSA) (Difco) and incubated for 24 h at 37 ºC. Listeria suspected colonies were identified by biochemical tests including Gram stain, motility, catalase, oxidase, β-haemolysis, carbohydrate fermentation tests (mannitol, rhamnose, xylose) and the Christie-Atkins-Munch-Peterson test (25).

DNA Isolation

A single *L. monocytogenes* colony was grown overnight on TSA at 37 ºC. One colony from the culture was inoculated into 5 ml of Tripticase Soy Broth (Difco) and incubated overnight at 37 ºC. The broth culture of 2.5 ml was then centrifuged, and the pellet was washed in 1 ml of distilled water and resuspended in an Eppendorf tube containing 400 µl of phosphate buffer saline (PBS). The tubes were vortexed and centrifuged at 11,600 x g for 5 min. The supernatant was discarded and the pellet was resuspended in 375 µl of STE buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 µl of 20 mg/ml Proteinase K and 20 µl of 10% SDS). The suspension was incubated at 55 ºC for 4 h, with vortexing every 30 min. An equal volume of phenol was added to the suspension, which was shaken vigorously by hand for 5 min and then centrifuged at 11,600 x g for 10 min. The upper phase was transferred into another Eppendorf tube. Genomic DNA was precipitated with absolute ethanol and 0.3 M sodium acetate at –20 ºC for 1 h. The mixture was then centrifuged at 11,600 x g for 10 min and the upper phase was discarded. The pellet was washed twice with 90% and 70% ethanol, respectively, with each step followed by 5 min centrifugation. The DNA pellet was gently resuspended in 200 µl of sterile distilled water.

RAPD analysis

In the RAPD analysis of *L. monocytogenes* strains, the reaction mixture was prepared in a total volume of 50 µl consisting of 5 µl of template DNA, 10x PCR buffer buffer (750 mM Tris-HCl, 200 mM $(NH_4)SO_4$, 0.1% Tween 20), 3.5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates, 1.25 U of Taq DNA polymerase (Fermentas, Lithuania), and 1 µM of OPA-11 primer (5'-CAA TCG CCG T –3'). The samples were amplified

through 50 cycles of denaturation (1 min at 94 ºC), primer annealing (1 min at 37 ºC), and chain extension (1 min at 72 ºC). A last step of extension was applied at 72 ºC for 10 min. Then 15 µl of PCR products were separated by electrophoresis in 1.5% agarose gels and visualised by ethidium bromide staining. For pattern analysis, 13 µl of the amplification products were loaded on 1.5% agarose gels and run at 70 V for 1 h. Gels were visualised by ethidium bromide and examined under ultraviolet light and photographed on Polaroid (type 667) film, and the patterns were compared visually (21).

Results

Isolation

L. monocytogenes suspected growth was observed in 10 samples in LSA after 48 h of incubation. They were identified as *L. monocytogenes* by biochemical tests and the isolation rate of *L. monocytogenes* was 6.6% from fish intestines.

RAPD analysis

The 10 isolates of *L. monocytogenes* were typed by RAPD analysis using a single strain oligonucleotide primer for epidemiological analysis. All isolates yielded positive results and band patterns with RAPD. Two different band profiles were obtained from the RAPD analysis of *L. monocytogenes* strains (Figure).

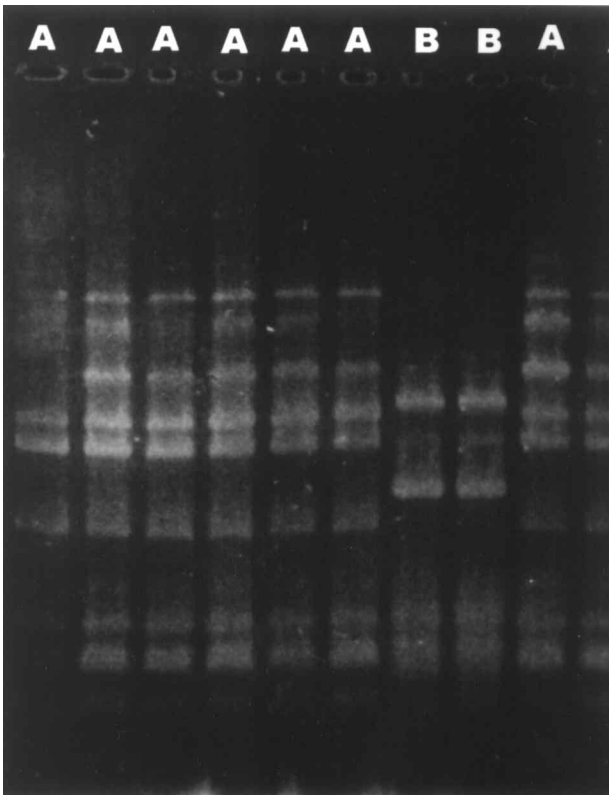

Figure. RAPD analysis of 10 *L. monocytogenes* isolates from fish intestines; A,B- profiles.

Discussion

Our results show that the fresh fish obtained from Keban Dam Lake contain *L. monocytogenes*, which is regarded as an important human pathogen, in their intestines. Intestinal contents of fish usually contaminate the fish meat and this causes the contamination of food-borne pathogens such as *L. monocytogenes* during the factoring procedure. The prevalence of *L. monocytogenes* in freshwater fish was 6.6%. This rate is lower than that in most of the published surveys on fresh fish.

Listeria spp. have been found in raw fish of freshwater and marine origins and *L. monocytogenes* was isolated from 62% of all water samples (26). In a study, 88 samples of salmon and salmon-trout were analysed for the presence of Listeria spp. and *L. monocytogenes*. The frequency of Listeria and *L. monocytogenes* in salmon was 12% and 0%, respectively, and in salmon-trout was 6.3% and 2.1% respectively (27). Miettinen et al. (28) investigated the surface contamination and the presence of *L. monocytogenes* in fish processing factories. Listeria spp. was determined at a rate of 45% and *L. monocytogenes* with at a rate of 12%.

In another study, 56 fresh fish samples were analysed for the presence of Listeria spp. Fifteen of the samples were found to be contaminated with Listeria spp. *L. monocytogenes* and *L. innocua* were isolated from 3 and 12 samples, respectively (29). Fuchs and Surendran (13), were unable to detect *L. monocytogenes* in fresh fish from India, although 33% of the samples harboured Listeria spp. However, Adesiyun (30) recorded a 2% incidence of *L. monocytogenes* in fish and shell fish in India.

The results of this study were lower than those of other, related studies. All fish used in this study were obtained from same area of the dam lake and the sewage system of the city had been discharging into this region for many years. Therefore the isolation rate was expected to be high. However, biological cleaning had been performed before discharging into the lake and this might

have caused the isolation rate to be lower than that in other reports.

The methods used for the isolation of Listeria from foods have varied. Most methods involve enrichment and selective plating. The media used for enrichment and selective plating have also varied (31). The method approved by the USDA was used for isolation of *L. myonocytogenes* in this study.

Among the molecular biological techniques, the RAPD method was chosen for this study due to several advantages, such as its greater discriminatory power, simplicity, reproducibility and sensitivity. In the analysis of the *L. monocytogenes* isolated from 10 samples by the RAPD method, 2 distinguishable and reproducible band profiles were obtained using a random primer (OPA-11). The different RAPD profiles raise the possibility that the isolated *L. monocytogenes* strains come from different sources. It was also indicated that there was no significant genetic difference between the isolates. With the addition of subsequently assayed random primers, the sensitivity and simplicity of the RAPD method could be improved for subtyping *L. monocytogenes* strains by exploiting the natural variability among individual isolates. Therefore many different random primers or other typing methods may be required for detailed analysis of isolates.

In conclusion, our results indicated that the fish living in Keban Dam Lake contain *L. monocytogenes* in their intestines and these fish may cause listeriosis outbreaks as reported previously (32,33). Humans eating the fish and their products are at risk of illness. The genotyping results showed that the strains isolated have different genetic profiles. This may be the result of different sources of bacteria. More discriminative typing techniques could be used to reveal the relationship between human, animal and fish isolates.

References

1. Jones, D., Seeliger, H.P.R.: The genus *Listeria*. In: Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K.H. (Eds.), 2nd Edition. The Prokaryotes – A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications, Vol. II. Springer-Verlag, New York, 1992; 1595-1616.
2. Colburn, K.G., Kaysner, C.A., Abeyta, C. Jr., Wekell, M.M.: *Listeria* spp. In a California coast estuarine environment. Appl. Environ. Microbiol., 1990; 56: 2007-2011.
3. Dijkstra, R.G.: The occurrence of *Listeria monocytogenes* in surface water of canals and lakes, in ditches of one big polder and in the effluents and canals of a sewage treatment plant. Zentralbl. Bakteriol. Mekrobiol. Hyg. B, 1982; 176: 202-205.
4. Farber, J.M., Peterkin, P.I.: *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev., 1991; 55: 476-511.
5. Lennon, D., Lewis, B., Mantell, C., Becroft, D., Dove, B., Farmer, K., Tonkin, S., Yeates, N., Stamp, R., Mickleson, K.: Epidemic perinatal listeriosis. Pediatr. Infect. Dis. J., 1984; 3: 30-34.
6. Baker, M., Brett, M., Short, P., Calder, L., Thornton, R.: Listeriosis and mussels. Commun. Dis. NZ., 1993; 93: 13-14.
7. Riedo, F.X., Pinner, R.W., Tosca, M., Carter, M.L., Graves, L.M., Reaves, M.W., Plikaytis, B.D., Broome, C.V.: A point source foodborne listeriosis outbreak: documented incubation period and possible mild illness. J. Infect. Dis., 1994; 170: 693-696.
8. Farber, J.M.: *Listeria monocytogenes* in fish products. J. Food Protect., 1991; 54: 922-934.
9. Guyer, S., Jemmi, T.: Behavior of *Listeria monocytogenes* during fabrication and storage of experimentally contaminated smoked salmon. Appl. Environ. Microbiol., 1991; 57: 1523-1527.
10. Fuchs, R.S., Reilly, P.J.A.: In: The Incidence and Significance of *Listeria monocytogenes* in Seafoods. Elsevier, Amsterdam, 1992; 217-229.
11. McLauchlin, J., Nichols, G.L.: Listeria and seafood. PHLS Microbiol. Digest., 1994; 11: 151-154.
12. Huss, H.H., Benembarek, P.K., Jeppesen, V.F.: Control of biological hazards in cold smoked salmon production. Food Cont., 1995; 6: 335-340.
13. Fuchs, R.S., Surendran, P.K.: Incidence of Listeria in tropical fish and fishery products. Lett. Appl. Microbiol., 1989; 9: 49-51.
14. Jeyasekaran, G., Karunasagar, I., Karunasagar, I.: Incidence of Listeria spp. in tropical fish. Int. J. Food Microbiol., 1996; 31: 333-340.
15. Seeliger, H.P.R., Hohne, K.: Serotyping of *Listeria monocytogenes* and related species. In: Bergan, T., Norris, J.R. (Eds.), Methods in Microbiology. Academic Press, New York, 1979; 31-49.
16. Schuchat, A., Swaminathan, B., Broome, C.V.: Epidemiology of human listeriosis. Clin. Microbiol. Rev., 1991; 4: 169-183.
17. Bibb, W.F., Gellin, B.G., Weaver, R., Schwartz, B., Plikaytis, B.D., Reeves, M.W., Pinner, R.W., Broome, C.V.: Analysis of clinical and food-borne isolates of *Listeria monocytogenes* in the United States by multilocus enzyme electrophoresis and application of method to epidemiologic investigations. Appl. Environ. Microbiol., 1990; 56: 2133-2141.
18. Nocera, D., Bannerman, E., Rocourt, J., Jaton-Ogay, K., Bille, J.: Characterization by DNA restriction endonuclease analysis of *Listeria monocytogenes* strains related to the Swiss epidemic of listeriosis. J. Clin. Microbiol., 1990; 28: 2259-2263.

19. Graves, L.M., Swaminathan, B., Reeves, M.W., Hunter, S.B., Weaver, R.E., Plikaytis, B.D., Schuchat, A.: Comparison of ribotyping and multilocus enzyme electrophoresis for subtyping of *Listeria monocytogenes* isolates. J. Clin. Microbiol., 1994; 32: 2936-2943.

20. Wagner, M., Maderner, A., Brandl, E.: Random amplification of polymorphic DNA for tracing and molecular epidemiology of Listeria contamination in a cheese plant, J. Food Protect., 1996; 59: 384-389.

21. Wernars, K., Boerlin, P., Audurier, A., Russell, E.G., Curtis, G.D.W., Herman, L., van der Mee-Marquet, N.: The WHO multicenter study on *Listeria monocytogenes* subtyping: random amplification of polymorphic DNA (RAPD). Int. J. Food. Microbiol., 1996; 32: 325-341.

22. Mazurier, S.I., Wernars, K.: Typing of Listeria starins by random amplification of polymorphic DNA (RAPD). Res. Microbiol., 1992; 143: 499-505.

23. Byun, S.K., Jung, S.C., Yoo, H.S.: Random amplification of polymorphic DNA typing of *Listeria monocytogenes* isolated from meat. Int. J. Food. Microbiol., 2001; 69: 227-235.

24. Jørgensen, L.V., Huss, H.H.: Prevalence and growth of *Listeria monocytogenes* in naturally contaminated seafood. Int. J. Food Microbiol., 1998; 42: 127-131.

25. Seeliger, H.P.R., Jones, D.: Genus Listeria. In: Kandler, O., Weiss, N. (Eds.), Regular, Nonsporing Gram-positive Rods. Bergey's Manual of Systematic Bacteriology, 2nd Ed. Williams and Wilkins, Baltimore, 1986; 1235-1245.

26. Dillon, R., Patel, T.: Listeria in seafoods: a review. J. Food Protect., 1992; 55: 1009-1015.

27. Vaz-Velho, M., Duarte, G., Gibbs, P.: Occurrence of Listeria spp. in salmon-trout (*Oncorhynchus mykiss*) and salmon (*Salmo salar*). Food Sci. Techol. Int., 1998; 4: 121-125.

28. Miettinen, H., Aarnisalo, K., Salo, S., Sjoberg, A.M.: Evaluation of surface contamination and the presence of *Listeria monocytogenes* in fish processing factories. J. Food Protect., 2001: 64: 635-639.

29. Vaz-Velho, M., Duarte, G., McLauchlin, J., Gibbs, P.: Characterization of *Listeria monocytogenes* isolated from production lines of fresh and cold-smoked fish. J. Appl. Microbiol., 2001; 91: 556-562.

30. Adesiyun, A.A.: Prevalence of Listeria spp., Campylobacter spp. and toxigenic *Escherichia coli* on meat and seafoods in Trinidad. Food Microbiol., 1993; 10: 395-403.

31. Karunasagar, I., Karunasagar, I.: Listeria in tropical fish and fishery products. Int. J. Food Microbiol., 2000; 62: 177-181.

32. Miettinen, M.K., Siitonen, A., Heiskanen, P., Haajanen. H., Bjorkroth, K.J., Korkeala, HJ.: Molecular epidemiology of an outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* in cold-smoked rainbow trout. J. Clin. Microbiol., 1999; 37: 2358-2360.

33. Ericsson, H., Eklöw, A., Danielsson-Tham, M.L., Loncarevic, S., Mentzing, L.O., Persson, I., Unnerstad, H., Tham, W.: An outbreak of listeriosis suspected to have been caused by rainbow trout. J. Clin. Microbiol., 1997; 35: 2904–2907.