SHORT COMMUNICATION

Veterinary Research Forum. 2022; 13 (1) 133 – 139
doi: 10.30466/vrf.2020.121715.2865
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Pulsed-field gel electrophoresis fingerprinting of *Listeria monocytogenes* isolates recovered from foods of animal origin and fishes in North-Eastern India

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Article Info

Article history:

Received: 15 February 2020
Accepted: 03 August 2020
Available online: 15 March 2022

Keywords:

Food
Genetic diversity
India
Listeria monocytogenes
Pulsed-field gel electrophoresis

Abstract

Listeria monocytogenes is a pathogen of great concern to the food industry. The present study was aimed to explore the clonal relationships amongst *L. monocytogenes* strains isolated from foods of animal origin (milk, beef, chevon (goat meat), pork and chicken) and fish. Forty-seven *L. monocytogenes* strains were characterized by pulsed-field gel electrophoresis (PFGE). The PFGE analysis using *ApaI* and *AscI* enzymes revealed 37 pulsotypes, with Simpson's discriminatory index of 0.987. This study demonstrated the presence of a few similar *L. monocytogenes* pulsotypes in different foods of animal origin in different places and years of isolation and this indicates that some *L. monocytogenes* subtypes may be ubiquitous which are acclimatizing and persisting in different foods of animal origin. This also emphasizes the importance of cross-contamination in local wet markets. Thus, the understanding of genetic diversity will contribute to the development of rational and workable strategies to control this important zoonotic infection.

Introduction

Among the genus *Listeria*, *L. monocytogenes* is a species of public health importance. It causes a food-borne disease primarily in immune-compromised individuals, causing septicemia and meningitis and in pregnant women; it may cause preterm delivery, mis-carriage or stillbirth.[1] With ingesting *L. monocytogenes* cells, healthy adults may endure pyretic gastroenteritis.[2] On the contrary to its low prevalence, the mortality rate of *L. monocytogenes* is relatively high.[3] The *L. monocytogenes* is a ubiquitous bacterium of both terrestrial and aquatic habitats eventually gaining access to various foods of animal origin and fishes.

Prevalence of *L. monocytogenes* has been reported across India in foods of animal origin and fishes[4-8] but, limited studies had been carried out to measure the genetic relatedness of *L. monocytogenes* isolates from different animal origin foods and fish.[9] Knowledge of epidemiology is indispensable in combating this serious foodborne pathogen. Most importantly, understanding the molecular ecology of the bacterial strains isolated from different food products and the whole food chain is vital in controlling listeriosis.[10]

The DNA-based genotyping techniques such as randomly amplified polymorphic DNA- polymerase chain reaction (PCR),[11] repetitive element sequence-PCR[12] enterobacterial repetitive intergenic consensus-PCR[12] and pulsed-field gel electrophoresis (PFGE)[9] have been utilized to a larger extent to establish epidemiological relationships between *L. monocytogenes* isolates from different sources. Among various typing approaches, PFGE has been considered to be the gold standard technique owing to its high reproducibility and discriminatory abilities.[13] With these understandings, the present study was envisaged to decipher the genetic relationship of the *L. monocytogenes* isolated from milk, pork, chevon, chicken, beef and fish using PFGE as a molecular typing tool. Such information can facilitate understanding the circulation of certain strains within different food commodities and the possibility of their inter-matrices transmission or contamination.

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Materials and Methods

Bacterial strains. The standard culture of *L. monocytogenes* (ATCC 19118) was obtained from Himedia Laboratories; India and *L. monocytogenes* (MTCC 1143) was procured from Microbial Type Culture Collection Centre, Institute of Microbial Technology, Chandigarh, India. Both strains were used as reference strains. The field strains included in this study were isolated from different foods of animal origin and fish from the North Eastern part (Meghalaya, Assam and Nagaland) of India. The isolates were already characterized and main-tained in our laboratory (Division of Animal Health, ICAR Research Complex for NEH Region, Umiam, Meghalaya, India). A total of 47 *L. monocytogenes* isolates from raw cow milk (n = 10), pork (n = 11), chevon (n = 12), chicken (n = 10), beef (n = 1) and fish (n = 3) were used in this study (Table 1). Before processing, all the test strains of *L. monocytogenes* were confirmed by bio-chemical characterization, PCR[14] and Phoenix™ 100 automated ID/AST system (Becton and Dickinson, Tuas Avenue, Singapore) as per the manufacturer's protocol.

Table 1. Sources and serotypes of *L. monocytogenes* strains used in this study.

Isolate No.	Source	Place of isolation	Serotypes	Year of isolation
2	Pork	Assam	1/2b, 3b	2012
3	Pork	Assam	4b, 4d, 4e	2012
4	Fish	Nagaland	1/2a, 3a	2012
5	Fish	Meghalaya	1/2a, 3a	2012
6	Pork	Assam	1/2b, 3b	2012
7	Fish	Assam	1/2a, 3a	2012
8	Pork	Assam	4b, 4d, 4e	2012
9	Pork	Meghalaya	1/2a, 3a	2012
10	Pork	Nagaland	1/2a, 3a	2012
11	Pork	Assam	4b, 4d, 4e	2012
12	Pork	Meghalaya	1/2a, 3a	2012
16	Chevon	Assam	1/2a, 3a	2012
17	Chicken	Assam	1/2a, 3a	2012
18	Chicken	Meghalaya	1/2a, 3a	2012
19	Chicken	Assam	1/2 b, 3b	2012
20	Chicken	Assam	1/2b, 3b	2012
21	Beef	Meghalaya	1/2a, 3a	2011
22	Milk	Meghalaya	1/2b, 3b	2011
23	Milk	Meghalaya	1/2a, 3a	2011
25	Milk	Meghalaya	1/2a, 3a	2011
27	Milk	Assam	4b,4d,4e	2013
28	Milk	Assam	1/2a, 3a	2013
29	Milk	Assam	1/2a, 3a	2013
30	Milk	Assam	1/2a, 3a	2013
32	Milk	Assam	1/2a, 3a	2013
33	Milk	Assam	1/2a, 3a	2013
34	Chicken	Assam	1/2a, 3a	2013
35	Chicken	Assam	1/2a, 3a	2013
36	Chicken	Assam	1/2a, 3a	2013
38	Pork	Assam	1/2a, 3a	3013
39	Pork	Assam	1/2b, 3b	2013
40	Pork	Meghalaya	1/2b, 3b	2012
41	Chevon	Assam	1/2a, 3a	2013
47	Milk	Assam	1/2a, 3a	2012
48	Chevon	Assam	1/2b, 3b	2012
50	Chicken	Meghalaya	1/2a, 3a	2013
51	Chicken	Meghalaya	1/2a, 3a	2013
52	Chicken	Meghalaya	1/2a, 3a	2013
53	Chevon	Meghalaya	1/2a, 3a	2013
54	Chevon	Meghalaya	1/2a, 3a	2011
55	Chevon	Meghalaya	1/2b, 3b	2011
56	Chevon	Assam	1/2b, 3b	2011
57	Chevon	Meghalaya	1/2a, 3a	2011
58	Chevon	Assam	1/2b, 3b	2013
59	Chevon	Assam	1/2a, 3a	2013
60	Chevon	Meghalaya	1/2a, 3a	2013
61	Chevon	Assam	1/2a, 3a	2013

Genomic DNA extraction. The isolated *L. monocytogenes* was retrieved from glycerol stocks (20.00%), kept at – 20.00 ˚C and cultured on nutrient agar (HiMedia Labs, India). The colonies of each strain were inoculated into 5.00 mL of brain heart infusion broth (HiMedia Labs, Mumbai, India). The inoculated tube was incubated at 37.00 ˚C for 24 hr. The genomic DNA was extracted using bacterial DNA extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Pulsed-field gel electrophoresis typing. The PFGE was performed following the Centres for Disease Control and Prevention PulseNet standardized procedure.[15] The PFGE typing was carried out in a CHEF-DRII apparatus (Bio-Rad Laboratories, Des Plaines, USA). The sample plugs were prepared by mixing 400 µL of bacterial cell suspension with 20.00 µL of lysozyme (20.00 mg mL^{-1}) solution (Sigma, St. Louis, USA) and incubated at 56.00 ˚C for 20 min. Sample plugs were digested with or 160 - 200 U of *ApaI* (New England Biolabs Inc., Ipswich, USA) at 30.00 ˚C for 5 hr and 25 U of *AscI* (Fermentas, USA) at 37.00 ˚C for 3 hr. The plugs were loaded and electrophoresed on 1.00% agarose gel (in 0.5X Tris-borate EDTA buffer; TBE) in the following conditions: Voltage, 6.00 V; Initial switch time, 4 sec; Final switch time 40 sec; Runtime 22 hr. The gels were then stained with 25.00 mL ethidium bromide (10.00 mg mL^{-1}) in 400 mL of 0.50x TBE for 30 min. The gels were visualized and photographed in the Gel Documentation System (Alpha Imager; ProteinSimple, California, USA) following destaining with deionised water (two washes of 20 - 30 min each using 400 mL).

The PFGE patterns generated by the typing method were analyzed using Phoretix 1D pro Gel Analysis Software (TotalLab, Newcastle, UK). The pictures were visually analysed and the restriction patterns were normalized against Lambda Ladder PFG Marker NO 340 S (New England BioLabs). The clustering was performed by an unweighted pair group algorithm and the dice correlation coefficient. The results of the clustering analysis were confirmed by a visual comparison of the PFGE profiles.

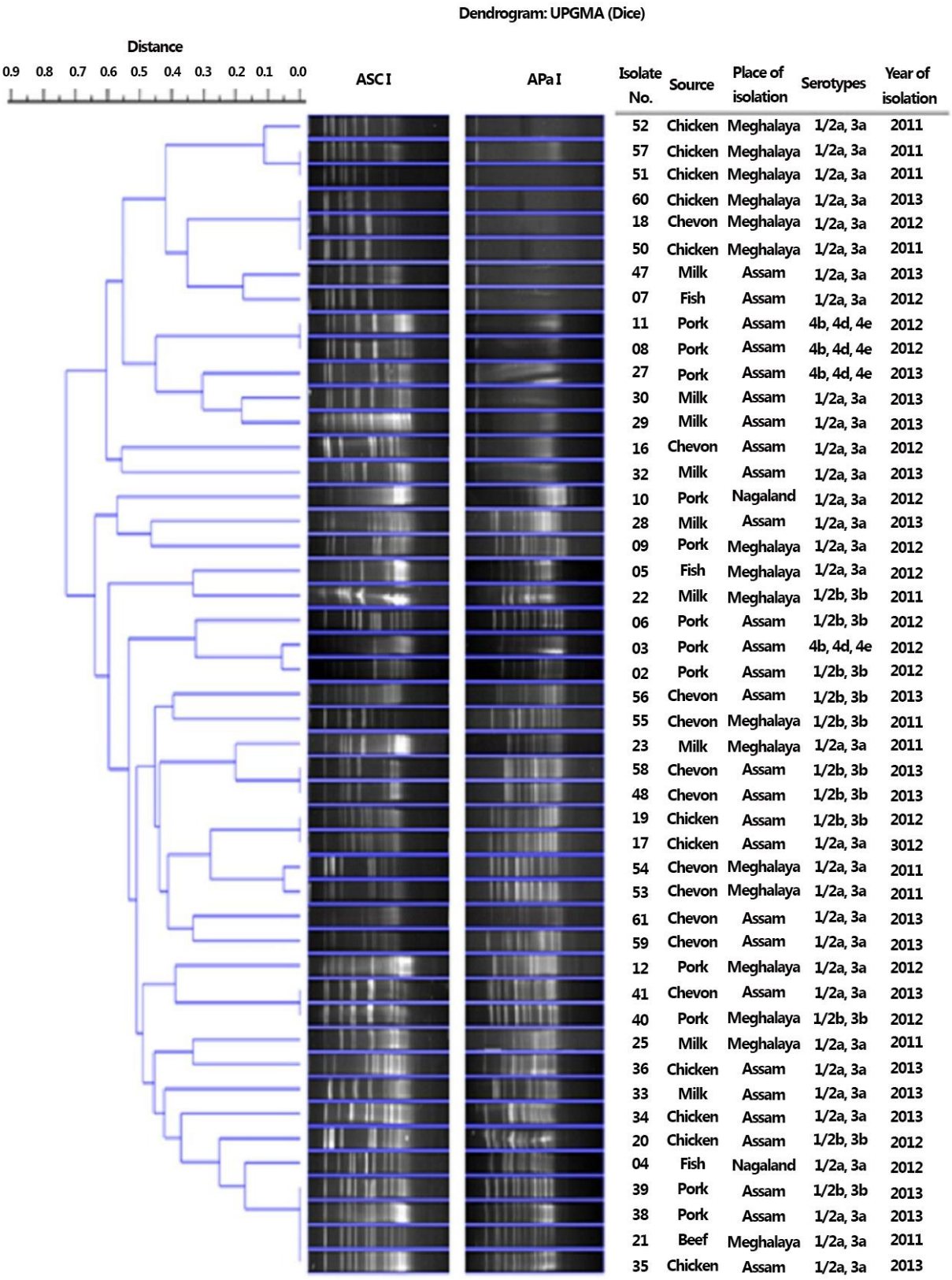
Results

The PFGE revealed a discriminative genetic profile for *L. monocytogenes* isolates from either similar or different sources (Figs. 1 and 2). In this present exercise, the PFGE analysis of 47 isolates using *ApaI* and *AscI* enzymes revealed 37 pulsotypes. The PFGE analysis of 47 test isolates using *ApaI* and *AscI* restriction enzymes distributed 17 test isolates into seven clusters (A-G); while, the remaining 30 isolates were unclustered (Figs. 1 and 2). Thirty-seven pulsotypes were observed with *ApaI- AscI* PFGE typing with a Simpson's discriminatory index (DI) of 0.987. Within the cluster, the isolates had a 100% similarity with each other. The PFGE distinction was also obvious between dissimilar serogroups, as most of the tested isolates fitting to the uniform serogroup were clustered together, regardless of their place, source and year of isolation.

Discussion

As far as India is concerned**,** listeriosis is not a notifiable disease. Besides that, the epidemiological data on this disease is not adequate in North East India, which is an important meat preferring belt. Our primary idea was to appreciate the diversity of this serious pathogen in different foods of animal origin in North-Eastern India. In the present study, 47 *L. monocytogenes* isolates recovered from different foods of animal origin from Assam, Meghalaya and Nagaland were typed and compared for their genotypic diversity. The PFGE has been successfully used in epidemiological investigations of *L. monocytogenes* earlier.[16] Since PFGE gets the benefit of restriction enzymes producing simple typing profiles (10 - 20 bands) by cutting DNA randomly. Moreover, analysis employing a computer is simple, allowing quick and effortless comparison of strains.[15] It is evident from our analysis that the DI value (0.987) exceeded the suggested value of 0.90.[17] Thus, typing employing both enzymes revealed a discriminative genetic profile for the *L. monocytogenes* isolates obtained from either similar or different food products. It shows that this amalgamation of restriction endonucleases, *Asc*I and *Apa*I gives excellent distinction for *L. monocytogenes* and banding patterns for both enzymes fall within the same size range.[15]

In the current experiment, PFGE analysis employing both enzymes revealed 37 pulsotypes which dispersed 47 *L. monocytogenes* isolates into seven clusters, A–G (2 to 4 isolates per cluster) possessing analogous fingerprint profile within their cluster. In cluster A, isolate 57, isolated from chevon was found to be identical to isolate 51 isolated from chicken and both these isolates were isolated from the same year and place (Meghalaya). In cluster B, isolates 60, 18 and 50 revealed identical fingerprints. Isolates 18 and 50 were isolated from chicken and 60 from chevon and the year of isolation was different. Moreover, all the isolates of clusters A and B fitted to similar serogroup (1/2a; 3a). Cluster C revealed an identical PFGE profile between two isolates (11 and 8) which are of similar serotype (4b; 4d and 4e), food product (pork), place (Assam) and year of isolation (2012). Similarly, cluster D showed a uniform PFGE profile between two isolates (58 and 48). Interestingly, the isolates of this cluster also have similar serogroup (1/2b and 3b), place (Assam), food matrix (chevon) and year of isolation (2013), suggesting that these isolates might have stemmed from a single source. However, in clusters E and F, isolates of different serogroups clustered together. In cluster E, isolate 19, isolated from the chicken was found



Isolate No.	Source	Place of isolation	Serotypes	Year of isolation
52	Chicken	Meghalaya	1/2a, 3a	2011
57	Chicken	Meghalaya	1/2a, 3a	2011
51	Chicken	Meghalaya	1/2a, 3a	2011
60	Chicken	Meghalaya	1/2a, 3a	2013
18	Chevon	Meghalaya	1/2a, 3a	2012
50	Chicken	Meghalaya	1/2a, 3a	2011
47	Milk	Assam	1/2a, 3a	2013
07	Fish	Assam	1/2a, 3a	2012
11	Pork	Assam	4b, 4d, 4e	2012
08	Pork	Assam	4b, 4d, 4e	2012
27	Pork	Assam	4b, 4d, 4e	2013
30	Milk	Assam	1/2a, 3a	2013
29	Milk	Assam	1/2a, 3a	2013
16	Chevon	Assam	1/2a, 3a	2012
32	Milk	Assam	1/2a, 3a	2013
10	Pork	Nagaland	1/2a, 3a	2012
28	Milk	Assam	1/2a, 3a	2013
09	Pork	Meghalaya	1/2a, 3a	2012
05	Fish	Meghalaya	1/2a, 3a	2012
22	Milk	Meghalaya	1/2b, 3b	2011
06	Pork	Assam	1/2b, 3b	2012
03	Pork	Assam	4b, 4d, 4e	2012
02	Pork	Assam	1/2b, 3b	2012
56	Chevon	Assam	1/2b, 3b	2013
55	Chevon	Meghalaya	1/2b, 3b	2011
23	Milk	Meghalaya	1/2a, 3a	2011
58	Chevon	Assam	1/2b, 3b	2013
48	Chevon	Assam	1/2b, 3b	2013
19	Chicken	Assam	1/2b, 3b	2012
17	Chicken	Assam	1/2a, 3a	3012
54	Chevon	Meghalaya	1/2a, 3a	2011
53	Chevon	Meghalaya	1/2a, 3a	2011
61	Chevon	Assam	1/2a, 3a	2013
59	Chevon	Assam	1/2a, 3a	2013
12	Pork	Meghalaya	1/2a, 3a	2012
41	Chevon	Assam	1/2a, 3a	2013
40	Pork	Meghalaya	1/2b, 3b	2012
25	Milk	Meghalaya	1/2a, 3a	2011
36	Chicken	Assam	1/2a, 3a	2013
33	Milk	Assam	1/2a, 3a	2013
34	Chicken	Assam	1/2a, 3a	2013
20	Chicken	Assam	1/2b, 3b	2012
04	Fish	Nagaland	1/2a, 3a	2012
39	Pork	Assam	1/2b, 3b	2013
38	Pork	Assam	1/2a, 3a	2013
21	Beef	Meghalaya	1/2a, 3a	2011
35	Chicken	Assam	1/2a, 3a	2013

Fig. 1. Pulsed-field gel electrophoresis (PFGE) profiles of *Listeria monocytogenes* isolates. The PFGE analysis using *ApaI* and *AscI* enzymes revealed 37 pulsotypes.

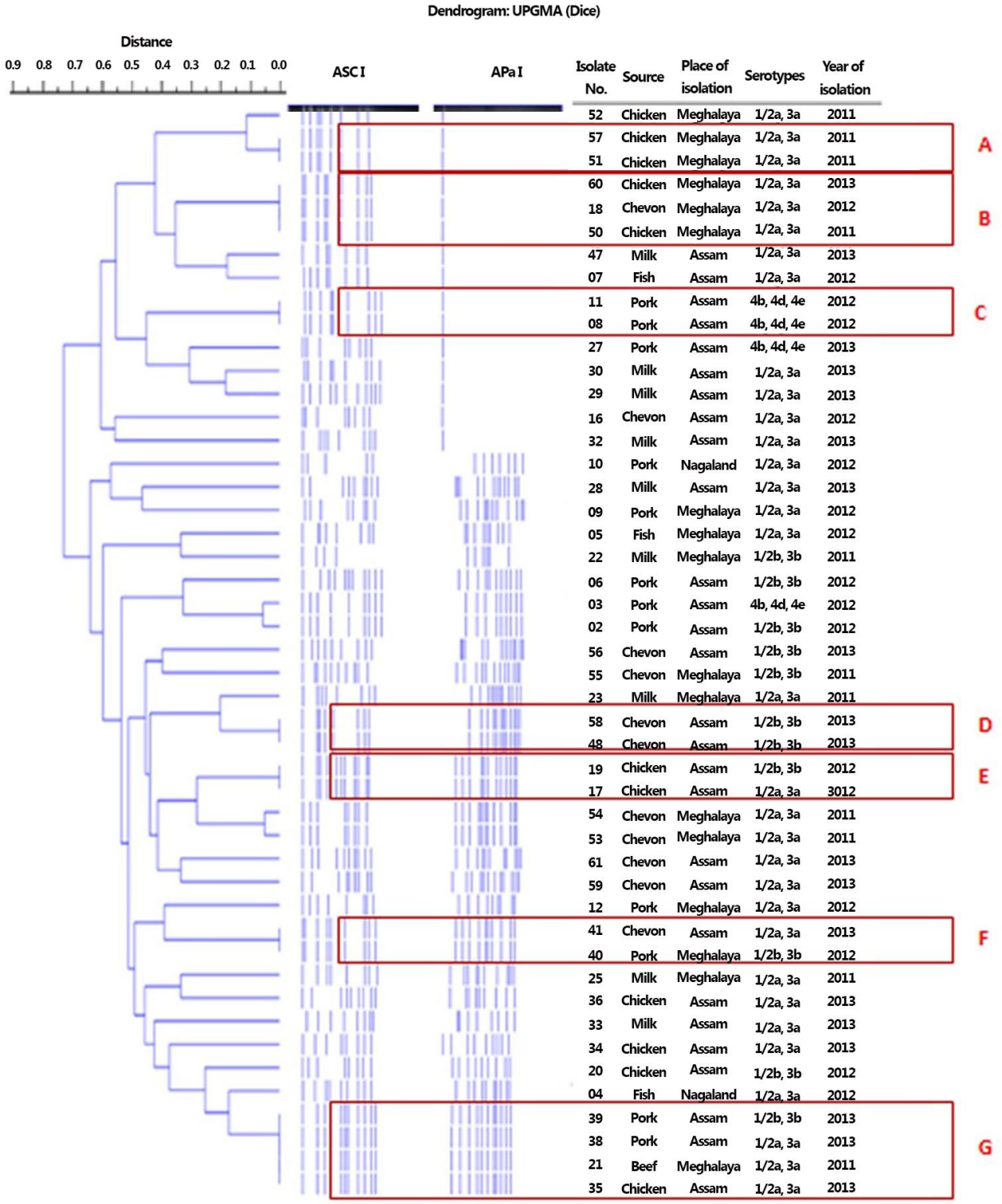


Fig. 2. Pulsed-field gel electrophoresis (PFGE) profiles of *Listeria monocytogenes* isolates. The PFGE analysis using *ApaI* and *AscI* enzymes revealed 37 pulsotypes which distributed 47 *L. monocytogenes* isolates into 7 clusters, A–G having similar fingerprint profile within their cluster.

to be identical to isolate 17 isolated from the chicken and both these isolates were isolated from the same year and place (Assam); although, the serogroups of the isolates were different. Notably, in cluster F, isolates 41 and 40 shared a similar PFGE pattern; although, their place, source and year of isolation were different. Moreover, both test isolates belonged to different serogroups. The experiment was also repeated to confirm the same. Cluster G is the bigger one with four isolates. It grouped isolates 39, 38, 21 and 35. Isolates 39 and 38 were from pork. Isolates 21 and 35 were isolated from beef and chicken, respectively. All the isolates belong to serogroup 1/2a;3a except isolate 39, which was from 1/2b;3b. Thus, from the above observations, some correlations were perceived between fingerprint profiles and serogroups. Interestingly, similar PFGE profiles were obtained from different foods of animal origin and places of isolation. This indicates cross-contamination in local wet markets and suggests that similar clones are being circulated in this region. It is also worthy to note that isolates of similar fingerprint patterns have been recovered in different years and similar pulsotypes have been witnessed in different foods of animal origin of the same place. This emphasises that some *L. monocytogenes* subtypes may be ubiquitous which are acclimatizing and persisting in different foods of animal origin. Our study showed that the same *L. monocytogenes* clones were found in different foods of animal origin such as pork, beef, chicken and chevon. This is concordant with previous reports indicating that similar isolates can be found in different food types from the same and different geographical areas and periods.[18-20]

In conclusion, our study suggested that similar *L. monocytogenes* subtypes are being circulated among different foods of animal origin in different places of isolation. It is also to be noted that isolates belonging to different serotypes can form a similar banding pattern. Such type of genotypic diversity throws better light on epidemiological tracking, surveillance and outbreak investigation. Our observation also suggests PFGE as an efficient and sensitive molecular typing tool for *L. monocytogenes* of different origins.

Acknowledgments

The authors are thankful to the Department of Biotechnology, Ministry of Science and Technology, New Delhi, India, for sanctioning the project (Research Grant No.: BT/01/CEIB/11/VI/13) and providing financial support. The authors are also grateful to the Director, ICAR RC for NEH Region, Umiam, Meghalaya, India, for providing the necessary facilities to conduct this work.

Conflict of interest

The authors declare that they have no conflict of interest.

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