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Characterization of *Listeria monocytogenes* isolates from food animal clinical cases: PFGE pattern similarity to strains from human listeriosis cases

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Abstract

Twenty-one isolates of *Listeria monocytogenes* from food animal clinical cases that involved meningitis or meningoencephalitis, encephalitis, mastitis and abortion were characterized by serotyping and pulsed-field gel electrophoresis (PFGE) in order to improve our understanding of the genetic links between individual strains and strains recovered from human listeriosis cases. Results showed that five of the isolates were serotype 1/2a, six were 1/2b, nine were 4b, and one was untypeable. A caprine, two bovine and an ovine brain isolate shared identical PFGE patterns indicating that strains of *L. monocytogenes* are not host specific. Other isolates exhibited distinct patterns that were not shared, indicating a genetic diversity. Dendrogram analysis revealed that PFGE patterns of the isolates clustered primarily according to serotype. We compared the PFGE types obtained for these isolates with PFGE types for human clinical isolates present in the CDC national PulseNet database. Six (29%) of the twenty-one strains had patterns that were indistinguishable from pathogenic human isolates in the database. Our observations offer preliminary evidence that food animals could be significant reservoirs of *L. monocytogenes* that lead to human infections and support the inclusion of PFGE patterns of veterinary clinical isolates in the national PulseNet database for increased surveillance.

Keywords: *Listeria monocytogenes*; Pulsed-field gel electrophoresis; PulseNet database; Serotyping

1. Introduction

Listeria monocytogenes is an important human and animal pathogen. The disease conditions in humans include septicemia, meningitis (or meningoencephalitis), encephalitis and spontaneous abortion or stillbirth [1]. In infected animals, pathogenic strains of *L. monocytogenes* can induce disease conditions similar to those observed in humans. Most infected animals do not survive, leading to substantial economic loss to food animal industries. Studies have implicated contaminated foods such as cheese, milk and beef in the transmission of the bacteria to humans [2–8]. Despite these observations, clinical isolates of *L. monocytogenes* from food animals have been very minimally characterized. Moreover, whether the source

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of contamination in the food chain is due to pre-harvest or post-harvest is still a matter of debate.

Thirteen different serotypes of *L. monocytogenes* have been identified to date. Whilst the majority of clinical isolates from epidemics or human sporadic cases belong to serotypes 1/2a, 1/2b, and 4b, other serotypes have been isolated occasionally from human listeriosis cases [2,9,7,10].

Serotyping and pulsed-field gel electrophoresis (PFGE) techniques have been successfully used in the characterization of *L. monocytogenes* [11]. However, the isolates used in those studies were cultured primarily from humans with listeriosis, non-symptomatic animals, and from the environment. There is little information on *L. monocytogenes* isolates recovered from food producing animals exhibiting clinical signs of listeriosis. PFGE method is standardized, reliable and reproducible, and as such is useful when conducting comparative genetic analysis and for molecular subtyping of *L. monocytogenes* isolates [12,11]. Comparisons between human and animal isolates may provide greater insight concerning the transmission of *L. monocytogenes* along the food chain, and the role of food animals as potential sources of human infection.

Recently, a national database of PFGE patterns that reside on a PulseNet server at the Centers for Disease Control and Prevention (CDC) in Atlanta, USA, was developed for foodborne bacterial pathogens including *L. monocytogenes*. PulseNet is a network of public health laboratories that performs DNA fingerprinting on bacteria that may be foodborne. The network identifies and labels each fingerprint pattern and permits rapid comparison of these patterns through an electronic database to identify related strains. Laboratories certified by CDC follow standardized protocols and have direct access to the PulseNet server to compare PFGE patterns for epidemiological investigations [13]. This network and database are essential in the emerging discipline of microbial forensics and is being implemented in the United States, Canada, Europe and Japan.

This study was undertaken primarily for two reasons. First, to subtype pathogenic isolates of *L. monocytogenes* recovered from food animal species using the CDC PulseNet standardized protocol in order to determine genetic links between individual strains and second, to use the PFGE data to search the PulseNet database to determine relatedness between the animal clinical isolates and isolates recovered from human with listeriosis.

2. Materials and methods

2.1. Bacterial isolates and serotyping

The *L. monocytogenes* strains used in this study are listed in Table 1. They were recovered as pure cultures from animal clinical cases submitted to the University of Wisconsin Veterinary Diagnostic Laboratory between August 2001 and November 2003. ScottA (taken as epidemic clone) and EGD strains were obtained from Dr. Charles Czuprynski, University of Wisconsin, Madison for direct comparison. All isolates were biochemically characterized by standard procedures, and serotyped on the basis of somatic (O) and flagella (H) antigen by a commercial kit according to the manufacturer's instruction (Listeria O Antisera "Seiken", Oxoid).

2.2. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis was carried out following the CDC standardized PulseNet protocol for *L. monocytogenes* with *Asc*I and *Apa*I as the restriction endonucleases [14]. The PFGE patterns were analyzed using the BioNumerics software package (version 3.0; Applied Maths). The TIFF images were normalized by aligning the peaks of the size standard strain (*Salmonella enterica* serovar Braenderup H9812 or *L. monocytogenes* H2446), which was loaded on three lanes in each gel, with the database global standard. Matching and dendrogram UPGMA (unweighted pair group method with averages) analysis of the PFGE patterns was performed using the Dice coefficient with a 1.0–1.5% tolerance window.

3. Results and discussion

3.1. Serotypes

Results showed that whereas one strain was untypeable with existing reagents the serotype distributions of the other isolates were as follows: 1/2a ($n = 5$ strains), 1/2b ($n = 6$), and 4b ($n = 9$) (Table 1). Most human infections are caused by strains of *L. monocytogenes* belonging to these three serotypes. Thus, the results indicated that the same serotypes that represent the majority of human listeriosis cases also are the cause of listeriosis in the food animal species. Non-typeable isolates of *L. monocytogenes* using the existing reagents have been reported previously [15]. We also isolated a non-typeable strain from a clinically ill animal (Table 1), indicating the existence of potential medically important strains that belong to serotypes other than the thirteen recognized serotypes within the species. Serotyping by slide agglutination assay takes advantage of the structural differences on the cellular (O) and flagella (H) antigens of *L. monocytogenes*. The differences contribute to reactivity and specificity of major group defining determinants. Thus, the non-typeable strain may exhibit differences on the structural arrangement of these antigens which are the target for the serotyping reagents

Table 1
Isolate number, source, serotype and origin of the *L. monocytogenes* including summary of clinical signs exhibited by the infected animals

Isolate number	Source	Animal species	Serotype	Clinical history/comment
W-820558	Milk	Bovine	4b	Mastitis
W-832504-2	Brain	Caprine	4b	Head tilt. Five deaths within a few days
W-832504-1	Brain	Caprine	4b	Head tilt. Dead within a few days
W-834256	Brain	Bovine	4b	Running in circle. Drooling. Temperature of 103 °F (39.4 °C). Dead
W-838293-1	Lung	Bovine	1/2b	Aborted fetus. 7% Abortion in herd
W-838293-2	Stomach	Bovine	1/2b	Aborted fetus. 7% Abortion in herd
W-840397	Brain	Bovine	4b	Animal could not swallow. Died in a few days
W-840850	Brain	Ovine	4b	Head tilt and trapped in corner. Died within 24 h
W-873094	Placenta, intestine, liver & lung	Bovine	1/2a	Third abortion in six months
W-873593	Brain	Ovine	NT	Animal circling to left. Temperature of 106 °F (41.1 °C). Went to recumbency with head held back to the right. Right ear drooping, quivering of lips. Animal euthanized after two days
W-875085	Stomach content	Bovine	4b	Aborted fetus. Three abortions in 1 month
W-888345	Brain	Bovine	1/2b	Poor balance, trouble bending neck. Animal died eight days from onset of illness
W-888844	Brain	Bovine	4b	Two day history of incoordination, head tilt. Animal died shortly after exhibition of clinical signs
W-889936	Brain	Bovine	4b	Animal exhibited central nervous system (CNS) signs, was circling to the right, agitated, ataxia, and progressed to recumbency within 48 h
W-889937	Brain	Bovine	1/2b	Extensive salivation, agitated, temperature of 103 °F (39.4 °C) and mild pneumonia. Animal went 1/2 through headlocks within 24 h acting wild. Died three days from onset of disease
W-896842	Lung	Bovine	1/2b	Calf sick immediately after birth. Animal died soon after. Five to six death with the same scenario
W-896964	Lung	Bovine	1/2b	Calf bloated at eight days after birth in fever. Slight pulmonary congestion. Animal died six days later
W-901015-3	Milk	Bovine	1/2a	Mastitis
W-904971	Milk	Bovine	1/2a	Mastitis
W-906895	Liver, lung, kidney, spleen & stomach content of fetus	Bovine	1/2a	Three abortions in two weeks. All abortions in last trimester
W-908024	Milk	Bovine	1/2a	Mastitis
ScottA	Human		4b	Human food-borne (milk) disease outbreak
EGD	Rabbit		1/2a	Rabbit outbreak

NT, non-typeable.

thus rendering the reagents non-reactive. Alternatively, serotyping can yield negative results if the strain failed to express the antigens in detectable amounts.

3.2. Pulsed-field gel electrophoresis

The similarity between the disease conditions presented by the infected animals to the conditions seen in humans with listeriosis such as elevated body temperatures, meningitis (or meningoencephalitis), and spontaneous abortion (Table 1) prompted us to hypothesize that the *L. monocytogenes* isolates from the food animal clinical cases might be linked to isolates associated with human listeriosis. We tested this hypothesis by using the pulsed-field gel electrophoresis technique to investigate the restriction pattern similarity of the animal isolates in relationship to one another and by using the CDC national PulseNet database on *L. monocytogenes*

strains from documented human listeriosis outbreaks to determine relatedness of the animal clinical isolates to isolates involved in human outbreaks. For data interpretation, the fingerprints generated by PFGE were considered indistinguishable on the basis of exact numbers and matching positions of all bands following *Asc*I and *Apa*I digestion, respectively. A single band difference was used to differentiate between strains and to assign a specific profile. This criterion was based on the assumption that identical strains will produce identical restriction fragment pattern regardless of the restriction endonuclease used for the DNA digestion (same genotype) but non-identical strains will produce non-matching banding pattern when different restriction endonucleases are used (different genotype). For many species, comparative studies indicated that isolates that are indistinguishable by PFGE are unlikely to demonstrate substantial differences by other typing methods [16–18]. In this study, when isolates recovered from different tissues of an animal exhibited identical patterns, the tissues were considered to have been infected by the same strain and in such instance one of the strains was used for analysis. Results of PFGE separation of *Asc*I macrorestriction fragments are shown in Fig. 1. We noted that a caprine, two bovine and an ovine brain strain which were isolated from clinically unrelated animals remote from one another both in time and in place shared identical patterns (Fig. 1; lanes 3, 8, 11, and 12), suggesting that they are related and that *L. monocytogenes* can cross animal species. Two milk isolates from different animals (Fig. 1; lanes 20 and 23) in the same herd exhibited identical patterns, also suggesting relatedness. Although epidemiological data is not available a common source of contamination could be the reason for the identity in both cases. Isolates recovered from different tissues (for example placenta, liver, lung and intestine) of a given animal, as in most abortion cases, yielded identical pattern indicating that the animal was infected by an invasive strain (data not shown). In cases where certain herds experienced a listeriosis outbreak (several animals affected), no pattern was found to be common to those isolates. All other isolates had distinct and unique profiles (Fig. 1), indicating different infecting strains and the existence of restriction pattern heterogeneity among animal clinical isolates of *L. monocytogenes*. These results are consistent with those obtained with *Apa*I

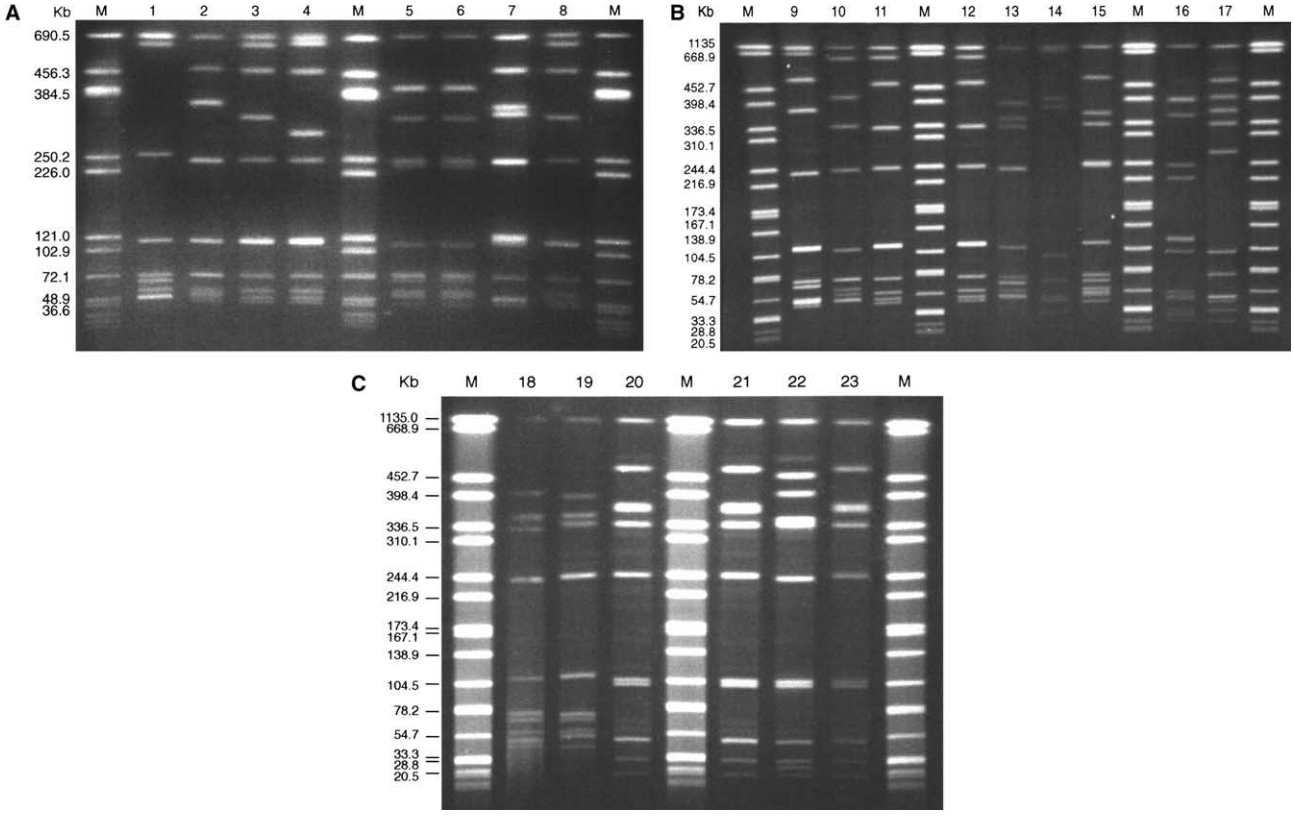

Fig. 1. PFGE separation of *Asc*I macrorestriction fragments of *L. monocytogenes* genomic DNA from animal clinical cases (see Table 1). Lanes: M, chromosomal DNA of *L. monocytogenes* H2446 digested with *Asc*I (A) and *S. enterica* serovar Braenderup H9812 digested with *Xba*I (B and C) as reference size markers in kilobase: 1, strain **W-820558**; 2, W-832504-2; 3, W-832504-1; 4, W-834256; 5, W-838293-1; 6, W-838293-2; 7, **W-840397**; 8, W-840850; 9, ScottA; 10, **W-888345**; 11, **W-888844**; 12, W-889936; 13, **W-889937**; 14, W-873593; 15, **W-875085**; 16, W-873094; 17, EGD; 18, W-896842; 19, W-896964; 20, W-901015-3; 21, W-904971; 22, W-906895; 23, W-908024. The strains identified in bold are those that were found to be indistinguishable from pathogenic *L. monocytogenes* in the CDC national PulseNet database.

macrorestriction (data not shown). PFGE using restriction endonucleases *Asc*I and *Apa*I was chosen for this study because the standardized protocol for *L. monocytogenes* typing of human isolates and a database of the PFGE patterns exists [14,13]. Further, it has been shown by several investigators that the PFGE method with this combination of restriction endonucleases has excellent discriminatory power with respect to the ability to clearly identify unrelated strains as being distinct from one another and results are easy to interprete [11]. Our observations were in agreement with these reports as the PFGE method provided excellent strain discrimination of the animal isolates used in this study compared with ribotyping (data not shown).

To determine whether the animal isolates are related to isolates from human listeriosis cases, we used the CDC national PulseNet database for comparative analysis of the PFGE subtypes of the animal and pathogenic human isolates. We found that six (29%) [W-820558, W-840397, W-875085, W-888345, W-888844, W-889937] of the twenty-one food animal clinical isolates used in this study had indistinguishable DNA fingerprints from strains isolated from documented human listeriosis outbreaks from varied geographical locations in the United States in the database suggesting that the isolates probably share a common ancestry. This observation supports our reasoning that food animals could be significant reservoirs of *L. monocytogenes* that lead to human infections and justify the inclusion of PFGE results from veterinary isolates in the CDC national PulseNet database as it will strengthen the surveillance effectiveness of the database.

Studies have implicated contaminated foods such as cheese, milk and beef in the transmission of *L. monocytogenes* to humans [2–8]. Similarly, animals can be infected through the consumption of contaminated feeds. The *L. monocytogenes* strains that are indistinguishable from pathogenic human strains were isolated from beef or dairy cattle and milk. Because food or feed sources produced in a region is often distributed to regions that are distant from one another one cannot rule out common source or path of exposure as a possible reason for some of the observed results. It is also safe to assume that some of the strains are clonal. In the absence of epidemiological data the reasons for the results are matter of speculation.

The PFGE molecular subtypes of the 21 *L. monocytogenes* strains including ScottA and EGD are presented in a dendrogram that utilized the PFGE data following *Asc*I digestion (Fig. 2). Results showed that isolates clustered primarily by serotype. PFGE distinguished four clusters of which the first contained all serotype 4b isolates including ScottA and a serotype 1/2b isolate with genetic similarities ranging from 80% to 100%. The second cluster contained serotype 1/2b strains with similarities of 85% to 100%. The third contained 1/2a strains including EGD with 62–100% similarities and the fourth consisted of the single non-typeable strain with 58% interlinkage homology (Fig. 2). ScottA and EGD are epidemic associated strains commonly used in laboratory research. While ScottA is a serotype 4b strain involved in a human milk outbreak, EGD is a serotype 1/2a strain involved in a rabbit outbreak. In a previous study *L. monocytogenes* strains were separated into three distinct lineages (lineages I, II, and III) based on ribotyping and allelic analysis of virulence genes. ScottA was found to belong to lineage I and EGD to lineage II. It was noted that the majority of the foodborne epidemic isolates as well as isolates from sporadic cases in humans and animals used in the study were closely related to ScottA (lineage 1) than to EGD (lineage II) [19]. Other investigators have also shown that *L. monocytogenes* can be divided into two major phylogenetic divisions in which division one contained serotype 1/2b and 4b and division II contained serotype 1/2a. Consistent with the results of a previous study [19], a significant association was found between division I (lineage I) and human strains, suggesting that strains from division I are better adapted to human hosts [20]. Based on the cluster analysis in this study, it is possible that most of our animal isolates mainly those of serotype 4b and some of 1/2b can cause disease in humans as they share close genetic relationship with ScottA (lineage I) and are indistinguishable from pathogenic human strains in the PulseNet database. Studies are underway to determine the scientific accuracy of this assumption. PFGE and serotyping showed that *L. monocytogenes* is heterogeneous serologically and genetically as observed herein. Cluster analysis suggested that even though there are differences genetically and in their somatic (O) and flagella (H) antigen types, strains belonging to a given serotype appear to be more closely related on the genetic level. The reason why most PFGE subgroups were within a single serotype is unclear. Correlations between molecular subtyping and serotyping of *L. monocytogenes* have been reported previously [21]. Other investigators have also attempted to define the connection between molecular subtypes and serotypes [12]. Although, there is currently no thorough knowledge of the molecular basis for the relationship between serotypes and molecular subtyping of *L. monocytogenes*, the presence of subset of fragments that are serotype specific have been suggested. A thorough and complete understanding of the genetic determinants of flagellar and somatic antigenic groups is required to elucidate the relationships between subtyping methods and to provide further insight into the evolution of the serotypes.

To our knowledge, this is the first report in which PFGE profile of veterinary clinical isolates was compared with the profile of pathogenic human isolates at the CDC national PulseNet database for correlation.

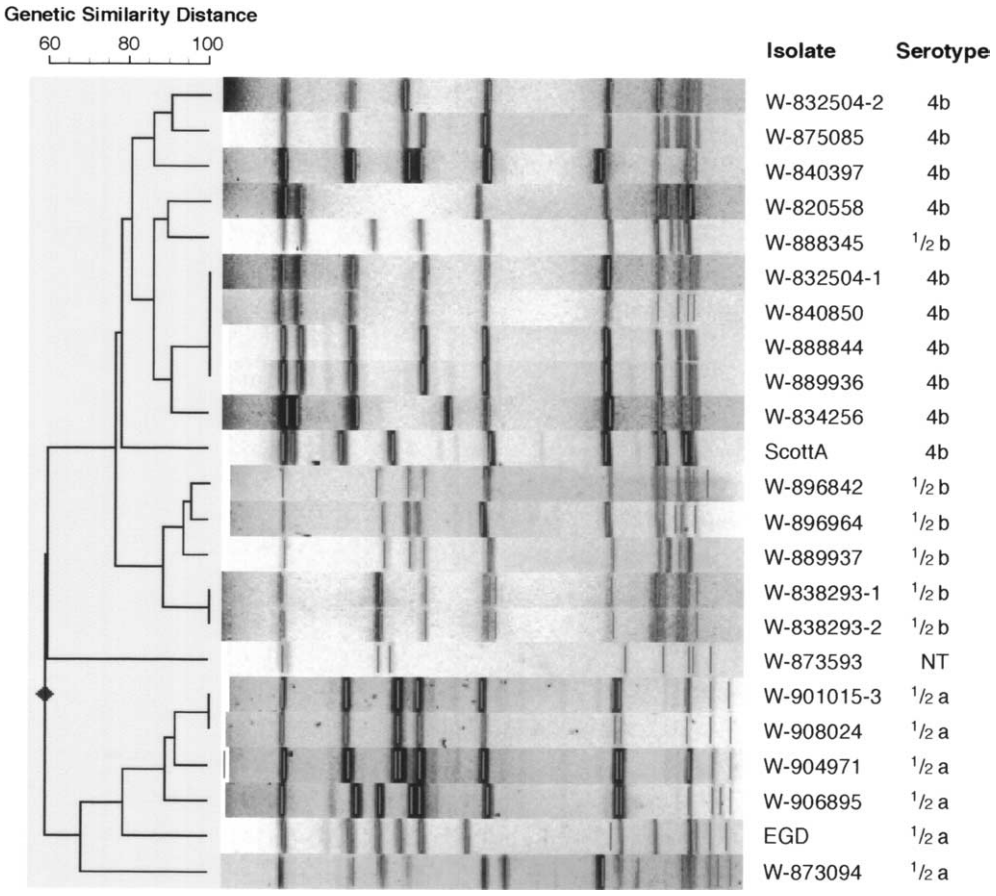

Fig. 2. Dendrograms derived from PFGE profile of *Asc*I macrorestriction showing restriction pattern similarity among the 21 *L. monocytogenes* animal clinical isolates, strains EGD and ScottA. Isolate identification numbers and serotype are indicated to the right side of the figure.

Because a limited number of strains were used, a more comprehensive approach that significantly increases the sample size will add strength to our observations. A better understanding of the ecological epidemiology and the genetic structure of listeria will be helpful in understanding the origin of animal and human listeriosis, which in turn will provide additional insight in the development of control strategies.

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