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Genetic characterization of *Listeria monocytogenes* from ruminant listeriosis from different geographical regions in the U.S.

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ABSTRACT

Listeria monocytogenes infections are an important disease of ruminants worldwide, causing encephalitis, septicemia, and abortions. Ruminant listeriosis can also pose a food safety risk due to the potential for *L. monocytogenes* to enter the food supply via the farm environment. Data on the genetic diversity of *L. monocytogenes* from ruminant clinical cases in the United States is limited. Our goal was to assess the genetic diversity of clinical listeriosis isolates from ruminants in the Upper Great Plains states, a population not well-studied, and compare this population to isolates from ruminants in New York State. Multi-locus sequence typing (MLST) was used to classify and compare the genetic diversity of the isolates from the two regions. Loci sequences were compared to all known sequence types using the Pasteur Institute *L. monocytogenes* MLST database. Four novel sequence types (ST) were identified among the Upper Great Plains isolates, and four new STs were classified in the New York collection. Five STs were found to be common across the 2 geographical regions; ST 1, 7, 191, and 204. Strains of ST 7 were most frequently isolated (7/46 isolates). Strains of ST 91 were all associated with fetal infections from the Upper Great Plains. Our results demonstrate that while there are some subtypes commonly found between the two geographic regions, there are also subtypes distinct to each region.

1. Introduction

Listeriosis is of major veterinary importance primarily in three farm ruminant species: cattle, sheep, and goats. Significant economic losses in livestock production occur due to the high morbidity and mortality in infected animals (Oevermann et al., 2010). Listeriosis manifests as either encephalitis or bacteremia in ruminants, and the septicemic form can lead to fetal infection and subsequent abortions. *L. monocytogenes* is acquired via oral transmission, and outbreaks of listeriosis in herds and flocks are often linked to consumption of contaminated silage (Garcia et al., 2016; Vazquez-Boland et al., 1992; Wiedmann et al., 1994). Additionally, asymptomatic carriage of *L. monocytogenes* in the gastrointestinal tract of ruminants allows the pathogen to multiply and then continue to circulate in the environment (Nightingale et al., 2004). Up to 50% of fecal samples collected from ruminants without clinical symptoms of listeriosis may contain *L. monocytogenes*, highlighting the potential for spread of the pathogen (Wesley, 1999). Others that have assessed the prevalence of *L. monocytogenes* on farms have detected this pathogen in 8–22% of water trough or tank samples, 11% of bedding samples, and 8 to 37% of farmyard soil samples, indicating the pathogen can be found throughout the farm environment (Dreyer et al., 2016; Garcia et al., 1996).

L. monocytogenes is also a human pathogen, causing symptoms similar to those seen in ruminants. Listeriosis in humans is also acquired through the oral route, with consumption of food contaminated with *L. monocytogenes* accounting for 99% of human cases (Scallan et al., 2011). The link between ruminant listeriosis and human listeriosis is not well understood, especially as direct transmission between ruminants and humans rarely occurs. However, ruminants may be an important natural reservoir for *L. monocytogenes* causing human infections, and the pathogen may then enter the human food supply via contamination of foods by manure or water (Oevermann et al., 2010).

Researchers have utilized sequence-based subtyping to characterize *L. monocytogenes* isolates from animal clinical cases, farms, foods, and human clinical cases (Bergholz et al., 2016; Chenal-Francisque et al., 2011; Haase et al., 2014). In an ovine listeriosis outbreak investigation, the same subtype was isolated from infected sheep as well as soil and water samples from the farm, which were considered as potential sources for the outbreak (Dreyer et al., 2015). In an examination of listeriosis isolates from cattle, sheep, and goats in Italy, researchers

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found that 12/20 isolates were of the same subtype, indicating circulation of that type in the region over time (Rocha et al., 2013). Subtyping of *L. monocytogenes* isolates from dairy herds and farms over time demonstrated that a few specific subtypes of the pathogen persisted over a 6-year period among herd members (Haley et al., 2015). The majority of information on *L. monocytogenes* subtypes in ruminants in the U.S. is from non-clinical isolates (Haley et al., 2015), or based on ribotyping, which is less commonly used than PFGE or MLST (Nightingale et al., 2004; Pohl et al., 2006). Our goal was to utilize multi-locus sequence typing (MLST) to characterize and compare the isolates causing listeriosis in ruminants from two geographic areas in the U.S., the Upper Great Plains (North Dakota, South Dakota, Minnesota) and New York State.

2. Methods

2.1. Isolates and DNA extraction

A total of 46 *L. monocytogenes* isolates were examined in this study, 19 from New York that were obtained from the Food Safety Lab at Cornell University, Ithaca, NY and have previously been subtyped by ribotyping (Pohl et al., 2006), and 27 from the Upper Great Plains that were provided by the North Dakota State Veterinary Diagnostic Lab (NDVDL), Fargo, ND. All isolates came from diagnosed clinical cases of listeriosis in ruminants (Table 1). Isolates were stored at −80 °C in brain-heart infusion (BHI) broth with 15% glycerol, and grown in BHI broth for 20 h prior to use for DNA extraction. DNA was extracted using either the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) or a modified phenol-chloroform protocol (Flamm et al., 1984). DNA quantity and quality was measured with a Nanodrop (Agilent, Santa Clara, CA). DNA with $A_{230}/A_{260} < 1.8$ were precipitated with 1/10 (vol/vol) 5 M sodium acetate and 2.5 (vol/vol) 100% ethanol, followed by suspension of the DNA in 10 mM Tris-EDTA. Only DNA with $A_{230}/A_{260} > 1.8$ was used for PCR.

2.2. Serotyping PCR

PCR-based serotyping was performed according to the protocol described by Kérouanton et al. (Kerouanton et al., 2010). This method consists of two PCR reactions, the first a multiplex with six primer pairs, five of which target specific genes for *L. monocytogenes* (lmo0737, lmo1118, *orf2819, orf2110, prfA*), and one primer pair specific for *Listeria* spp. (*prs*). A second PCR reaction was performed to target *flaA*, which encodes a flagellar protein present in *L. monocytogenes*. Amplified PCR fragments were separated by 2% agarose gel electrophoresis. Band patterns from the first multiplex PCR were used to classify the isolates into one of five serogroups, and the secondary *flaA* PCR was used to distinguish between serogroups IIa (1/2a, 3a) and IIc (1/2c, 3c).

2.3. MLST

MLST based on seven genes was used to classify the multi-locus genotype of each *L. monocytogenes* isolate. Amplification of the 7 loci (*abcZ*, *bglA*, *cat*, *dapE*, *dat*, *ldh*, and *lhkA*) was conducted using PCR according to Stessl et al. (Stessl et al., 2014). PCR reagents and concentrations were as follows: magnesium chloride (Promega, Madison, WI) at 2.5 mM, dNTPs (Promega) at 200 µM, 5X colorless buffer (Promega) at 1X, GoTaq (Promega) at 1U, and forward and reverse primers (Integrated DNA Technologies, Coralville, IA) at 200 nM each. PCR conditions were: an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 2 min. For amplification of *ldh,* the annealing temperature was reduced to 48 °C. Amplified products were visualized on a 1.5% agarose gel, and gel extraction was performed as needed (Omega Gel Extraction Kit, Omega Biotek, Norcross, GA). Amplicons were sequenced using universal primers (Ragon et al., 2008) at Macrogen, USA or McLAB, San Fransisco, USA. Strain information and sequence data are available at the Food Microbe Tracker database, www.foodmicrobetracker.com.

2.4. Sequence data analysis

Sequence data were analyzed using Geneious version 6.1 (Biomatters, Auckland, New Zealand). Acquired sequences were aligned and trimmed according to a reference sequence of the appropriate locus obtained from the Pasteur MLST database. A consensus sequence was generated for each locus of each strain, and sequences queried against the Pasteur Institute *Listeria monocytogenes* MLST database. The database was used to assign an allele number to each sequence, and the combination of allele numbers (allelic profile) determines the sequence type (ST). MEGA7 (Kumar et al., 2016) was used to construct a neighbor-joining consensus tree from the concatenated allele sequences, using the Jukes-Cantor model and 1000 replicates. ST 562 from lineage IV was chosen to be the root.

2.5. Statistical analysis

Fisher's exact test was used to determine significant associations between lineage of an isolate and the clinical manifestation of listeriosis in the ruminant. The number of isolates belonging to each lineage 1 and 2 were allocated to either neurologic infection, bacteremia, or fetal infection based on the clinical information provided from the NDVDL or published information on the isolate (Pohl et al., 2006). The three isolates that did not clearly belong to a lineage were excluded from the analysis. Fisher's exact test was implemented in SAS v. 9.4 (SAS Institute, Cary, NC).

3. Results

3.1. Novel STs identified

MLST was used to classify *L. monocytogenes* isolated from cases of ruminant listeriosis in New York State and the Upper Great Plains (UGP). A total of 26 STs were identified among the 46 isolates (Table 1). Of these 26 STs, 8 (31%) were novel STs that had not been previously identified in the Pasteur *Listeria* MLST database (Fig. 1). The 8 novel STs were each represented by a single isolate. Three of the novel STs were single allele variants of known STs, and were assigned to existing clonal complexes (CC). For example, the novel ST 1239 differs from ST 1 at the *abcZ* allele, and is a member of CC 1. Two of the novel STs are singletons, not members of a described CC. These are ST1282 in lineage 1 and ST1057 in lineage 2. Three of the novel STs form a distinct branch between lineage 2 and 3 and do not clearly belong to either lineage (Fig. 1). Notably, these three novel STs all originated from ruminants in North and South Dakota.

3.2. Diversity of STs over geographical regions

Overall, lineage 2 isolates (29/46, 63%) were more frequent in our dataset than lineage 1 isolates (14/46, 30%). The isolates from New York State were almost equally represented between lineage 1 (9/19 isolates) and lineage 2 (10/19 isolates), whereas lineage 2 was more frequently isolated from the UGP (19/27 isolates) compared to lineage 1 (5/27 isolates). Serotypes of isolates within each lineage were as expected, with isolates of serotypes 1/2b and 4b belonging to lineage 1 and isolates of serotypes 1/2a and 1/2c belonging to lineage 2 (Table 1). The majority of the New York State isolates had previously been ribotyped (Pohl et al., 2006) and lineage 2 isolates with the same ribotype tended to have the same ST. For example, isolates FSL E1-0042, J2-0007, and J2-0019 are all ribotype 1030A, and all belong to ST 7. Isolates FSL J2-0018 and J2-0011 are both ribotype 1039C, and both belong to ST 204. In contrast, lineage 1 isolates of the same ribotype did not have the same ST. For example, isolates FSL J2-0038,