Determination of Leukotoxin Structural Gene (LktA) of *Mannheimia haemolytica* Isolates from Bovine Pneumonia in Japan during 2010

Saiki Imamura[a]

Michiko Kawanishi[a]

Fumiya Hirano[a]

Ken Katsuda[b]

Kinya Yamamoto[a]

Mariko Uchiyama[a]

Hidetaka Nagai[a]

Mayumi Kijima[a]

[a] National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, 1-15-1, Tokura, Kokubunji, Tokyo 185-8511, Japan

b Pathology and Pathophysiology Research Division, National Institute of Animal Health, 3-1-5 Kannondai Tsukuba Ibaraki 305-0856, Japan

Corresponding author: Saiki Imamura; National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, 1-15-1, Tokura, Kokubunji, Tokyo 185-8511, Japan

E-mail: saiki_imamura@nm.maff.go.jp

Tel.: +81-42-321-1841; Fax: +81-42-321-1769

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ABSTRACT

This study was conducted to provide fundamental information on the capsular serotype and leukotoxin structural gene (lktA) genotypes of Mannheimia haemolytica field isolates from cattle with pneumonic pasteurellosis in Japan during 2010. Of the 54 isolates serotyped, 29 (53.7%) were serotype A1, 10 (18.5%) were serotype A2, 8 (14.8%) were serotype A6, and 7 (13.0%) were untypable via slide agglutination testing. Sequencing analysis revealed that the lktA1.1-type leukotoxin was exclusively associated with serotypes A1 and A6. In contrast, lktA2.1- and 8.1-type leukotoxins were associated with both serotype A2 and untypable isolates.

This study revealed that cattle infected with M. haemolytica in Japan were exposed to at least three types of leukotoxins. However, further comprehensive studies are required to investigate allelic variants of

Table 1. *Characterization of M. haemolytica used in this study.* [a]

BAC plate [b]	Phenotype					PCR
Growth	L-Arabinose	Sorbitol	Aesculin	Catarase	Oxidase	*lkt*
+	-	+	-	+	+	+

[a] *Results are summarized based on the 54 isolates from pneumonic pasteurellosis in Japan in 2010.*
[b] *Blood agar plates containing 15 ug/mL bacitracin.*

lktA in M. haemolytica prevailed in Japan.

INTRODUCTION

Mannheimia haemolytica is a complicating agent in bovine respiratory disease, and is recognized as an important pathogen in feedlot cattle (Rice et al, 2007). Based on capsular antigens or lipopolysaccharide complexes, there are 17 serotypes and numerous untypable strains of M haemolytica (Angen et al, 1999). Bovine pasteurellosis, primarily due to serotype A1 commonly manifests as severe fibrinous pleuropneumonia (Lillie, 1974).

M haemolytica leukotoxin is specifically virulent for ruminant lymphoid cells, and is a key factor in the pathogenesis of pneumonic pasteurellosis. Significant antibody responses to leukotoxins correlate with resistance to experimental M haemolytica challenge (Clinkenbeard et al, 1989; Highlander et al, 1989). However, the cytotoxic activity of M haemolytica strains depends on the diversity of the leukotoxin structural gene (lktA), which has a complex evolutional history and at least eight major allelic variants (Davies et al, 2001).

The identification of prevalent serotypes and allelic variants of lktA provides useful information for vaccine development. However, little is known about the distribution of lktA allelic variants in Japan. Thus, this study will investigate the prevalence of M haemolytica serotypes and allelic variants of lktA throughout Japan.

MATERIALS AND METHODS

M haemolytica Field Isolates

total of 54 M haemolytica isolates were obtained from different cattle with pneumonic pasteurellosis in 16 prefectures in Japan during 2010. The isolates were analyzed for catalase and oxidase activities, and positive samples were then identified via multiplex PCR (Alexander et al, 2008).

Serotyping of M. haemolytica Isolates from Bovine Pneumonia

Antisera were prepared in rabbits by intravenous inoculations of formalinized whole cell suspensions of reference strains (Biberstein, 1978; Fodor et al, 1988) of M haemolytica, provided by Dr. G.H. Frank, National Animal Disease Center, Ames, Iowa, USA. Briefly, two New Zealand rabbits were injected biweekly with an antigen of each serotype reference strain. Antisera were used to serotype isolates using a slide agglutination test (Frank and Wessman, 1978). However, antiserum against the newly described serotype A17 was not included in the test.

LktA Genotyping of M haemolytica Isolates from Bovine Pneumonia

PCR amplification and DNA sequence analysis were conducted based on the method reported by Davies et al (2001). Namely, the lktA gene was amplified from the chromosomal DNA with the 5' primer

Table 2. *Frequency of M. haemolytica isolates in Japan during 2010*

Serotype				Total number of isolates
A1	A2	A6	UT [a]	
Number of isolates (%) [b]				
29 (53.7)	10 (18.5)	8 (14.8)	7 (13.0)	54

[a] *Untypable*
[b] *Percentage of each serotype among the total isolates*

Table 3. *LktA-types of M. haemolytica isolates in Japan (n=32).*

Serotype	A1		A2		A6	UT [a]
lktA-type	A1.1	A2.1	A8.1	A1.1	A2.1	A8.1
Number [b]	14	2	4	8	1	3

[a] *Untypable*
[b] *Number of tested isolates*

lktA9 (5'-TCA AGA AGA GCT GGC AAC-3') and the 3' primer lktA7 (5'-AGT GAG GGC AAC TAA ACC-3'). PCRs were carried out in a thermal cycler (TAKARA, Tokyo Japan) using the following amplification parameters: denaturation at 94°C for 45 s, annealing at 62°C for 45 s, and extension at 72°C for 2 min. Thirty cycles were performed, and a final extension step of 72°C for 10 min was used. Production of a PCR amplicon of the expected size (~3 kbp) was confirmed by agarose gel electrophoresis, and the DNA was purified with a QIAquick PCR purification kit (Qiagen, Tokyo, Japan). Dye terminator cycle sequencing was performed with the Big Dye Terminator Cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA), and sequence reactions were performed and read by an automated DNA sequencer (ABI PRISM3130, Applied Biosystems). Both strands of the lktA gene were sequenced using seven internal primer pairs, designed as sequence data became available. Nucleotide sequence data were analyzed and edited with the GENETYX (version 9) program.

Nucleotide Sequence Accession Numbers

The GenBank accession numbers used in this study are lktA1.1 - AF314503, lktA2.1 - AF314511, and lktA8.1 - AF314515.

RESULTS

Frequency of M haemolytica Serotype in Japan During 2010

All 54 field isolates from cattle with pneumonic pasteurellosis in 2010 were phenotypically and genetically identified as M haemolytica (Table 1). Serotyping results are shown in Table 2. Of the 54 isolates serotyped, 29 (53.7 %) were serotype A1, 10 (18.5 %) were serotype A2, 8 (14.8 %) were serotype A6, and 7 (13.0 %) isolates were untypable.

LktA-types of M. haemolytica Isolates in Japan During 2010

Of the 54 isolates, 32 (14 serotype A1, 6 serotype A2, 8 serotype A6, 4 untypable isolates) were selected from different prefectures in order to increase geographic diversity. lktA allelic variants were analyzed by DNA sequencing (Table 3). Serotypes A1 and A6 all contained lktA1.1, and serotype A2 and untypable isolates contained either lktA2.1 or 8.1; 22 (68.8 %), 3 (9.4 %) and 7 (21.9 %) isolates produced lktA1.1-, 2.1-, and 8.1-type leukotoxins, respectively.

Alignment Comparison of Putative Amino Acid Sequences for lktA

Putative amino acid sequences of lktA were aligned to previously reported sequences (data not shown). These putative amino acid sequences had 100% identity to the reference sequences previously reported by Davies et al [6].

DISCUSSION

For many bacterial pathogens, identifications of serotype and virulence factor are useful in understanding outbreak epidemiology, monitoring of infected cattle, and preparing vaccines for disease control.

In accordance with serological survey done by Katsuda et al (2012), the majority (87%) of isolates were represented by only three serotypes (A1, A2 and A6). Serotypes A1 and A6 only used lktA1.1, while serotype A2 and untypable isolates used lktA8.1 or A2.1. Davies et al also reported that lktA1.1 was distributed among serotype A1 and A6 (Davies and Baillie, 2003). Though the lktA8.1-type leukotoxin is well known to be an ovine isolate (Davies, 1997), they appeared to be isolated from bovine with pneumonic pasteurellosis in Japan.

Strain selection for vaccine development is pivotal for disease protection. In

Japan, two commercial vaccines have been developed; both are from serotype A1 and have prevented disease in cattle, since the prevalence of M. haemolytica in Japan has remained relatively constant.

CONCLUSION

However, continuous determination of serotype and lktA allelic variants of field isolates would aid in determination of effective control measures against M haemolytica. As far as we know, this is the first report on information regarding capsular serotypes and the allelic variations of lktA in M haemolytica recently prevailed in Japan.

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