NOTE *Bacteriology*

First isolation of *Actinobacillus* genomospecies 2 in Japan

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ABSTRACT. We describe here the first isolation of *Actinobacillus* genomospecies 2 in Japan. The isolate was found in a septicemic foal and characterized by phenotypic and genetic analyses, with the latter consisting of 16S rDNA nucleotide sequence analysis plus multilocus sequence analysis using three housekeeping genes, *recN*, *rpoA* and *thdF*, that have been proposed for use as a genomic tool in place of DNA-DNA hybridization.

KEY WORDS: 16S rDNA, *Actinobacillus* genomospecies 2, horse, housekeeping gene, isolation

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Bisgaard taxon 9, which is isolated from the oral cavities of healthy horses as well as diseased horses with septicemia and arthritis, belongs to the genus *Actinobacillus sensu stricto* ("true actinobacilli") [1, 2]. This taxon has been separated into two novel species, *Actinobacillus arthritidis* and *Actinobacillus* genomospecies 2, based on 16S rDNA sequence analysis and DNA-DNA hybridization (DDH), although the two species cannot be phenotypically differentiated [2]. One or both species have been reported to be isolated from horses in the Czech Republic, Denmark, Australia, Sweden and Zimbabwe [2], but not in Japan. We report here the first isolation of *Actinobacillus* genomospecies 2 in Japan. The isolate was identified in a septicemic foal using both phenotypic and genetic analyses. The latter consisted of 16S rDNA nucleotide sequence analysis plus multilocus sequence analysis using three housekeeping genes, *recN*, *rpoA* and *thdF*, that can predict whole genome similarity and have been proposed for use as a genetic tool in place of DDH [6].

In early May 2007, a two-day-old foal died on a farm in Japan where 6 work horses and 118 beef cattle were being raised. For bacterial isolation, 5% defibrinated sheep Trypticase agar (Difco, Sparks, MD, U.S.A.) was used, and the agar inoculated samples were incubated at 37°C in the presence of 5% CO_2. Gram-negative rods were isolated in pure culture from various organs, including the brain and pharynx lymph nodes, as well as from ascites fluid, suggesting that the cause of death was septicemic infection due to insufficient colostrum feeding. One of the strains, strain KMM1, was isolated from the liver and was found to be non-hemolytic, catalase-positive and oxidase-positive and to grow in a nicotinamide dinucleotide-independent manner. The other biochemical characteristics of strain KMM1 were examined by using a biochemical identification kit, ID test HN20-Rapid (Nissui Pharmaceutical, Tokyo, Japan). The results are shown in Table 1. The seven-digit biochemical profile number generated by the ID test HN-20-Rapid was 7107373, resulting in 60% and 38% relative probability of being *Actinobacillus lignieresii* and *Actinobacillus pleuropneumoniae*, the hosts of which are ruminants/horses and pigs, respectively [1]. Since the host of isolation has been reported to be one of the important pieces of information for separating *Actinobacillus sensu stricto* at the species level and *A. pleuropneumoniae* is hemolytic [1], *A. pleuropneumoniae* could be ruled out. Consequently, strain KMM1 was tentatively identified as *Actinobacillus* genomospecies 1 (equine *A. lignieresii*) [2]. However, misidentification based on phenotypic characterization is a frequent and serious problem in the case of *Pasteurellaceae* family members [3]. In fact, there are no data for the equine pathogen, Bisgaard taxa 9 representing *A. arthritidis* and *Actinobacillus* genomospecies 2 [2], in the database of the biochemical identification kit. The biochemical characteristics of *A. arthritidis* CCUG 24862^T and *Actinobacillus* genomospecies 2 CCUG 15571 were therefore examined by using the ID test HN20-Rapid as a reference (Table 1). The results showed that the biochemical profiles of strain KMM1 and *A. arthritidis* CCUG 24862^T were identical. On the other hand, the only

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Table 1. Results of ID test HN-20 rapid

Biochemical tests	Strain KMM1	*Actinobacillus arthritidis* strain CCUG 24862^T	*Actinobacillus* genomospecies 2 strain CCUG 15571
Alanine aminopeptidase	+	+	+
Phosphatase	+	+	+
Nitrate reduction	+	+	+
Urease	+	+	+
Orthinine decarboxylase	–	–	–
Indole	–	–	–
Glucosidase	–	–	–
Proline aminopeptidase	–	–	–
γ-glutamyl aminopeptidase	–	–	–
Production of acid from:			
(+)-D-Glucose	+	+	+
(+)-D-Maltose	+	+	+
(−)-D-Fructose	+	+	+
(+)-D-Mannose	+	+	+
(−)-D-Mannitol	+	+	+
(+)-D-Trehalose	–	–	–
(+)-D-Sucrose	+	+	+
(+)-D-Lactose	+	+	+
(+)-D-Xylose	+	+	+
β-galactosidase	+	+	+
Nitrite reduction	+	+	–

difference between strain KMM1 and *Actinobacillus* genomospecies 2 CCUG 15571 was the presence or absence of nitrite reduction, which may not be an important phenotype for the separation of equine actinobacilli and which was not described in the previous reports [1, 2] (Table 1). The results suggested that strain KMM1 may be *A. arthritidis* or *Actinobacillus* genomospecies 2 in addition to *Actinobacillus* genomospecies 1 (equine *A. lignieresii*).

Genetic analysis was then performed in order to conclusively identify strain KMM1. First, the 16S rDNA nucleotide sequence, which is widely used in bacterial species descriptions, of strain KMM1 was determined as described elsewhere [5]. The 16S rDNA nucleotide sequence from strain KMM1 showed the highest identity to *Actinobacillus* genomospecies 2 (99.8% and 99.6%), followed by *Actinobacillus equuli* subsp. *haemolyticus* (98.1%), *Actinobacillus hominis* (98%) and *A. arthritidis* (97.9% and 97.7%) (Supplementary file 1). The threshold of 16S rDNA sequence similarity between two strains for separating them into different species has been reported to be over 97% [9] and later was reported to be still higher, within the range of 98.7–99.0% [8], due to the presence of higher similarities between closely related taxa. The results of the 16S rDNA analysis revealed that KMM1 is likely to be *Actinobacillus* genomospecies 2 as per a criterion with a higher sequence similarity value (98.7–99.0%), since almost of the *Actinobacillus sensu stricto* showed over 97.0% similarity (Supplementary file 1).

Many veterinary diagnosticians seem to generally and routinely identify clinical specimens by using only 16S rDNA sequence analysis. However, it has been generally accepted that other more sensitive methods with greater resolution, such as DDH or analysis of gene sequences, are required to separate two strains into different species when the 16S rDNA sequence similarity between two strains is over the threshold (97.0% or 98.7–99.0%) separating two species [8, 11]. DDH is still considered as a gold standard for species description, but is tedious and cumbersome. In addition, the genome DNA-DNA similarity values of DDH show a very high variation among experiments and among laboratories [6]. Therefore, a group known as the Ad Hoc Committee for the Re-evaluation of Species Definition in Bacteriology has suggested the development and validation of alternatives to DDH [10]. Among several studies for exploiting alternative methods that are highly reproducible and compatible with DDH, it has been reported that the nucleotide sequence similarity of three housekeeping genes—i.e., *recN* (encoding a DNA repair protein), *rpoA* (encoding the alpha subunit of the RNA polymerase) and *thdF* (encoding a GTPase)—between strains can be used to deduce whole genome DNA-DNA similarity, and it has therefore been proposed that these genes be used as an identification tool that is highly reproducible and compatible with DDH [6]. The nucleotide sequence of the housekeeping genes was determined as described elsewhere [6]. The nucleotide sequence similarity of the genes from strain KMM1 and type strains of each species were calculated using CLUSTALW in MegAlign Pro application included in lasergene 12 Core suit (DNASTAR, Madison, WI, U.S.A.). Whole genome DNA-DNA similarity values were then calculated using the formula described elsewhere [6]. Consequently, the whole genome similarity values of strain KMM1 calculated from the three housekeeping genes, *recN*, *rpoA* and *thdF,* were 90.7%, 84.7% and 68.2–71.3%, compared with those of *Actinobacillus* genomospecies 2, *A. arthritidis* and other species belonging to *Actinobacillus*

sensu stricto, respectively (Supplementary file 1). Since the threshold of the whole genome similarity value has been reported to be around 85% for species separation in the family *Pasteurellaceae* [6], strain KMM1 can be identified as *Actinobacillus* genomospecies 2.

The nucleotide sequences of 16S rDNA, *recN, rpoA* and *tdhF* were deposited in the DDBJ/EMBL/Genbank DNA databases under accession numbers AB493823, AB492088, AB492089 and AB492091, respectively.

Strain KMM1 could be genetically identified as *Actinobacillus* genomospecies 2, whereas the genetic methods are generally considered to be too expensive and cumbersome for the routine veterinary diagnostic laboratories (VDL), and only a limited number of VDL, such as reference laboratories, are familiar with the genetic methods. Therefore, there is need of an easy and cost-effective system for the non-genetic rapid identification of members of the family *Pasteurellaceae* isolated from clinical specimens of animals. Laymann *et al*. reported that the phenotypic bacterial identification system used by their VDL does not have a set of criteria for identifying *A. arthritidis,* and they therefore described that several of the unclassified *Actinobacillus* spp. that could not be speciated were likely to be *A. arthritidis* [7]. Likewise, *A. arthritidis* and *Actinobacillus* genomospecies 2 were not included in a routine phenotypic identification system that was developed to differentiate members of the family *Pasteurellaceae* isolated from animals [4]. Therefore, a routine phenotypic method for identifying *A. arthritidis* and *Actinobacillus* genomospecies 2 should be established.

In conclusion, strain KMM1, which was isolated from a diseased foal, was identified as *Actinobacillus* genomospecies 2 by using phenotypic analysis and genetic analyses consisting of 16S rDNA nucleotide sequence analysis plus whole genome DNA relatedness calculated from the nucleotide similarity of three housekeeping genes, *recN*, *rpoA* and *thdF*, which is highly reproducible and compatible with DDH. To the best of our knowledge, this is the first isolation of *Actinobacillus* genomospecies 2 in Japan.

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