



NOTE

Bacteriology

Actinobacillus species isolated from Japanese Thoroughbred racehorses in the last two decades

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ABSTRACT. *Actinobacillus* species are known to be pathogenic to horses. To clarify etiological agents of actinobacillosis in Japanese adult horses, 27 isolates from Japanese Thoroughbred racehorses putatively identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry as *Actinobacillus* were further identified by PCR of the *A. equuli* toxin gene, by CAMP test, and by 16S rRNA sequencing analysis. *Actinobacillus equuli* subsp. *haemolyticus* was isolated most frequently (16/27) and was related to respiratory infections. *Actinobacillus equuli* subsp. *equuli* (4/27) was isolated from chronic cases or concomitant with other bacterial infections. The remainder were *A. pleuropneumoniae*, unclassified *Actinobacillus* species and *Pasteurella caballi*. *Actinobacillus equuli* including subsp. *haemolyticus* and subsp. *equuli* were the species most frequently isolated from equine actinobacillosis in Japan.

KEY WORDS: 16S rRNA, *Actinobacillus*, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS), repeats-in-toxin (RTX), Thoroughbred

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Actinobacillus species are Gram-negative rod-shaped bacteria classified in the family *Pasteurellaceae* [17]. Although *Actinobacillus* species naturally reside in the mucous membranes of the respiratory tract and genitourinary tract of healthy animals, some species are thought to cause disease when they invade tissues following local trauma [17]. *Actinobacillus pleuropneumoniae* (*A. pleuropneumoniae*) in particular is known to be highly contagious and to cause fibro-hemorrhagic necrotizing pneumonia in pigs [21]. *Actinobacillus* species were known to be associated specific hosts, and phenotypic identification of *Actinobacillus* at species level is difficult without information on host of isolation [4].

Among *Actinobacillus* species in horses, the most frequently isolated are *Actinobacillus equuli*. (*A. equuli*) subspecies (subsp.) *equuli* or *A. equuli* subsp. *haemolyticus*; *Actinobacillus lignieresii* (*A. lignieresii*) and *A. pleuropneumoniae* are also occasionally isolated [15], although *A. pleuropneumoniae* might be misidentified since the isolates were identified only by phenotypic characteristics and is exclusively isolated from pigs [3]. These organisms are associated with various clinical manifestations in horses including respiratory infections, septicemia, metritis, mastitis, arthritis, endocarditis, meningitis, or stillbirth [14]. *Actinobacillus equuli* subsp. *equuli* is the etiological agent of “sleepy foal disease” which is an acute (sometimes chronic) type of fatal septicemia in neonatal foals [10]. *Actinobacillus equuli* subsp. *haemolyticus* has been reported to have caused fatal pulmonary hemorrhage in an adult horse [19]. Systemic actinobacillosis is thought to occur mainly in neonatal foals because of failure of transfer of passive immunity and conditions in the birthing environment [23]. Adult horses are rarely infected with *Actinobacillus* species unless some other disease or predisposing factor is present [22]. Although, Thoroughbred racehorses are bred under stresses including strict training, race or long transportation, for horses an etiological agent of actinobacillosis in this population is still not clear.

Toxins belonging to the repeats-in-toxin (RTX) family are produced by some species of *Actinobacillus*, and they exert hemolytic and cytotoxic activity [1, 12]. *Actinobacillus equuli* subsp. *haemolyticus* can be discriminated from *A. equuli* subsp. *equuli* by the presence of *A. equuli* toxin (Aqx), a member of the RTX toxin family, which is encoded by the *aqx* gene [14]. Furthermore, other *Actinobacillus* species are characterized by the existence of toxins of the RTX family [14]. *Actinobacillus pleuropneumoniae* RTX-toxin I (Apx I), Apx II, Apx III and ApxIV, which are encoded on polycistronic operons *apxICABD*, *apxIICA*, *apxIIICABD* and *apxIVA* respectively, play a role in the pathogenicity of *A. pleuropneumoniae* [20]. PCR assays based on *apx* genes have been used for identification and typing of *A. pleuropneumoniae* strains [25]. Production of Apx I and Apx II has been detected in *A. suis* [11], and *A. lignieresii* has *apxICABD* without the canonical promoter sequences and lacks expression of Apx I [20]. Analyzing these operons would help to identify *Actinobacillus* species.

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Table 1. Source of isolates used in this study and results of identification

Strain No.	Year of isolation	Age of host	Source	MALDI-TOF MS identification	Aqx	CAMP test	Final identification
NE-1	1996	4	Guttural pouch infection	<i>Actinobacillus equuli</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-26	2000	2	Shipping fever	<i>Actinobacillus equuli</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-28	2000	2	Shipping fever	<i>Actinobacillus equuli</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-29	2000	2	Shipping fever	<i>Actinobacillus suis</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-60	2000	2	Shipping fever	[<i>Actinobacillus</i>] <i>rossii</i>	+	-	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-72	2000	3	Pneumonia	<i>Actinobacillus equuli</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-75	2000	3	Pneumonia	<i>Actinobacillus equuli</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-77	2000	3	Pneumonia	<i>Actinobacillus equuli</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-87	2001	2	Pneumonia	<i>Actinobacillus equuli</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-109	2002	2	Guttural pouch infection	<i>Actinobacillus equuli</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-110	2002	3	Pneumonia	<i>Actinobacillus suis</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-111	2002	3	Pneumonia	<i>Actinobacillus equuli</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-178	2010	3	Pneumonia	<i>Actinobacillus suis</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-186	2011	4	Pneumonia	<i>Actinobacillus equuli</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-202	2013	3	Pneumonia	<i>Actinobacillus suis</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-238	2015	2	Abscess	<i>Actinobacillus equuli</i>	+	+	<i>Actinobacillus equuli</i> subsp. <i>haemolyticus</i>
NE-134 ^{a)}	2004	3	Thrombosis	<i>Actinobacillus equuli</i>	-	+	<i>Actinobacillus equuli</i> subsp. <i>equuli</i>
NE-155 ^{a)}	2008	4	Phlebitis	<i>Actinobacillus equuli</i>	-	-	<i>Actinobacillus equuli</i> subsp. <i>equuli</i>
NE-185 ^{a)}	2011	3	Endocarditis	<i>Actinobacillus equuli</i>	-	+	<i>Actinobacillus equuli</i> subsp. <i>equuli</i>
NE-240	2015	2	Stone bruise	<i>Actinobacillus equuli</i>	-	+	<i>Actinobacillus equuli</i> subsp. <i>equuli</i>
NE-146 ^{a)}	2006	3	Pneumonia	<i>Actinobacillus pleuropneumoniae</i>	-	-	<i>Actinobacillus pleuropneumoniae</i>
NE-159	2009	3	Lymphadenitis	<i>Actinobacillus pleuropneumoniae</i>	-	-	<i>Actinobacillus pleuropneumoniae</i>
NE-2 ^{a)}	1996	4	Colitis	<i>Actinobacillus pleuropneumoniae</i>	-	-	<i>Actinobacillus</i> sp.
NE-23	1999	3	Pneumonia	<i>Actinobacillus lignieresii</i>	-	-	<i>Actinobacillus</i> sp.
NE-144 ^{a)}	2005	3	Pneumonia	[<i>Actinobacillus</i>] <i>rossii</i>	-	-	[<i>Pasteurella</i>] <i>caballi</i>
NE-147	2006	3	Pneumonia	[<i>Actinobacillus</i>] <i>rossii</i>	-	-	[<i>Pasteurella</i>] <i>caballi</i>
NE-239	2015	2	Stone bruise	[<i>Actinobacillus</i>] <i>rossii</i>	-	-	[<i>Pasteurella</i>] <i>caballi</i>

a) Isolated from a necropsied case. MALDI-TOF MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; Aqx, *A. equuli* toxin.

To clarify etiological agents of actinobacillosis in adult race horses, we characterized *Actinobacillus* species isolated from Japanese Thoroughbred racehorses.

The 27 bacterial isolates used in this study were obtained from 27 Thoroughbred racehorses (9 were 2 years old, 14 were 3 years old and 4 were 4 years old) between 1996 and 2015 (Table 1) and were kept at -80°C until use. Of the 27 cases, 6 cases (NE-2, NE-134, NE-144, NE-146, NE-155, and NE-185) were necropsied cases and the other 21 were clinical cases (Table 1).

The isolates were putatively identified as *Actinobacillus* species by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) performed on a MALDI Biotyper (Bruker Japan, Yokohama, Japan) according to the manufacturer's instruction with database version 8.0.0.0. The species listed under MALDI-TOF MS in Table 1 are the best-match species presented by MBT Compass software (Bruker Japan).

The isolates identified by MALDI-TOF MS were then examined by analysis of the 16S rRNA sequence analysis, RTX gene PCR and CAMP activity as follows. 16S rRNA sequencing analysis was performed in accordance with published methods [8, 9] by sequencing 1,500 bp PCR products amplified with primers: 27F, 5'-AGAGTTTGATCMTGGCTCAG-3' and 1525r, 5'-AAGGAGGTGATCCAGCC-3'. The obtained sequences were compared with published 16S rRNA gene sequences by using EZbiocloud (<https://www.ezbiocloud.net>) [24] and identified according to the guidelines of the Clinical and Laboratory Standards Institute [6]. The isolates were identified in the species level when the obtained sequences matched the reference sequence of type strain with over 99.0% identity and greater than 0.8% separation from other species, or identified in the genus level when the sequences matched the reference or valid sequences with 97.0–98.9% identity. To determine the presence of the RTX gene, Aqx-specific PCR based on the *aqx* gene, or Apx I-IV-specific PCR based on the presence of *apxI-IV* genes were performed as described previously [1, 7, 25], using bacterial genomic DNAs extracted by InstaGene Matrix (Bio-Rad, Hercules, CA, U.S.A.). For analysis of CAMP activity, a CAMP test using a *Staphylococcus aureus* streak on a sheep blood agar plate was performed. The isolates were finally identified comprehensively according to the results of MALDI-TOF MS identification, 16S rRNA sequence analysis, PCR of RTX gene or CAMP test gene sequencing.

The final identification of each isolate is described in Table 1. Of the 27 isolates, 16 (59.3%) were identified as *A. equuli* subsp. *haemolyticus* which were *aqx*-positive and 4 (14.8%) were *A. equuli* subsp. *equuli* which were *aqx*-negative. Of the remaining isolates, 2 (7.4%) were *A. pleuropneumoniae*, 2 (7.4%) were identified as *Actinobacillus* species but unclassified at the species

level and 3 (11.1%) were *Pasteurella caballi*. With the 16S rRNA sequence analysis, all the isolates except for 3 isolates identified as [*Pasteurella*] *caballi* had high similarity with multiple *Actinobacillus* species or with under 99% identity. With regard to the MALDI-TOF MS identification shown in Table 1, the results for some isolates in this study were inconsistent from the final identification. Four isolates (NE-29, NE-110, NE-178, and NE-202) were identified as *Actinobacillus suis* (*A. suis*) with MALDI-TOF MS; however, they were identified as *A. equuli* subsp. *haemolyticus* in the final identification. Three isolates (NE-144, NE-147, and NE-239) were identified as *Pasteurella caballi* with 16S rRNA sequencing, even though they were identified as [*Actinobacillus*] *rossii* with MALDI-TOF MS. Although MALDI-TOF MS is reported to be able to discriminate *Actinobacillus* species except for closely related species such as subspecies of *A. equuli* [13], these conflicting results seem to reflect the limitation of MALDI-TOF MS identification in these genera. For identification of isolates in *Pasteurellaceae*, other identification methods would be needed together with MALDI-TOF MS identification.

Four isolates (NE-2, NE-23, NE-146, and NE-159) were not identifiable at the species level by 16S rRNA sequencing analysis. With the 16S rRNA sequencing analysis, 3 isolates (NE-2, NE-146, and NE-159) had lower than 99% identity with *Actinobacillus* species; NE-2 matched *A. hominis* with 97.6% identity, NE-146 matched *A. lignieresii* with 98.89% identity, and NE-159 matched *A. lignieresii* with 98.6% identity. NE-23 had high percent identity with two different *Actinobacillus* species; matched *A. lignieresii* with 99.2% identity and *A. pleuropneumoniae* with 99.0% identity. As reported previously [4], 16S rRNA sequencing analysis might have some difficulty in identifying certain *Actinobacillus* species. With the PCR of RTX, four isolates were *aqx*-negative and only *apx*IBD-positive, which suggested that these isolates had the operons *apxI*. NE-146 and NE-159 were also *apxIV*-positive, therefore they were identified as *A. pleuropneumoniae*. Further studies are needed for final identification of the strains that could not be identified in this study.

Of the 16 isolates identified as *A. equuli* subsp. *haemolyticus*, 9 isolates were isolated from cases with pneumonia, 4 were isolated from cases with shipping fever, 2 were isolated from cases with guttural pouch infection, and 1 was isolated from an abscess. In this study, *A. equuli* subsp. *haemolyticus* was the most frequently isolated from Thoroughbred racehorses in *Actinobacillus* species, and most of them were isolated from the respiratory tract. Recent study in the United States reported that *A. equuli* subsp. *equuli* was the most frequently isolated from adult horses [15], which showed different result from this study. Although *A. equuli* subsp. *haemolyticus* is thought to be part of the normal flora of the equine oral cavity and has been isolated from tracheal washes [14], this species is also reported as a cause of pneumonia in young adult horses [3]. It is reported that the diseases caused by this species usually occur together with other factors, such as viral infection or stresses resulting from training [3], which is a situation that might have occurred in our cases.

Isolates identified as *A. equuli* subsp. *equuli* in this study were derived from horses with thrombosis, phlebitis, endocarditis, or stone bruise. Although *A. equuli* subsp. *equuli* is known to be a pathogen of newborn foals, it is often isolated from adult horses [15]. In this study, *A. equuli* subsp. *equuli* was isolated from 4 young adult horses, 3 of which were necropsied cases that might have had another chronic disease or bacterial infection before infection with *A. equuli* subsp. *equuli*. The case from which NE-134 was isolated had been treated earlier for colitis before finally being euthanized because of his poor condition. During the necropsy, not only *A. equuli* subsp. *equuli* but also *Escherichia coli*, *Staphylococcus xylosum*, *Staphylococcus aureus*, and *Streptococcus equi* subsp. *zooepidemicus* were isolated from the thrombus (data not shown). The other 2 necropsied cases were euthanized because of poor condition following acute laminitis (case of NE-155) or endocarditis (case of NE-185), and *A. equuli* subsp. *equuli* was the sole bacterium isolated from these 2 cases. In adult horses, *A. equuli* infections occur rarely or are limited to cases with some other concomitant disease or predisposing factor [18, 22]. Our cases from which *A. equuli* subsp. *equuli* was isolated might each have suffered from another disease before *Actinobacillus* infection.

Actinobacillus species that produce RTX toxin show co-hemolytic (CAMP) activity towards erythrocytes [12]; however, some of the isolates in this study showed a contradiction between the results of the PCR of *aqx* analysis and the CAMP tests. One of the *aqx*-positive isolates (NE-60) did not show CAMP activity, and 3 isolates (NE-134, NE-185, and NE-240) were CAMP positive without possessing the *aqx* gene. The same phenotype has been reported for an *Actinobacillus equuli* strain isolated from a rabbit, in which there was a positive reaction in the CAMP test but where the *aqx* gene was not detected by PCR because of a DNA mutation in the primer binding site [16]. The isolates in our study might need further research to elucidate the reason for contradiction between *aqx* PCR and CAMP test in this study. Furthermore, *aqx*-positive CAMP test-negative isolate (NE-60), which was identified as [*A.*] *rossii* with MALDI-TOF MS, might be the variant of *A. equuli* subsp. *haemolyticus*.

Two isolates were identified as *A. pleuropneumoniae* (NE-146 and NE-159) and two isolates (NE-2 and NE-23) were unclassified *Actinobacillus* species, and there was no consistency about their source or year of isolation. Even though these 4 isolates were not able to be identified by 16S rRNA sequence analysis, 3 of them (NE-23, NE-146 and NE-159) had high similarity with *A. lignieresii* or *A. pleuropneumoniae*. *A. lignieresii* or *A. pleuropneumoniae*-like species have previously been isolated from horses: *A. lignieresii* has been isolated from the oral cavity of horses [5], and is reported to be a pathogen of equine cellulitis [2], and *A. pleuropneumoniae*-like species has been isolated from barren horses and horses with osteomyelitis [15]. In order to accurately discuss the pathogenicity of these *Actinobacillus* species, it will be necessary to isolate them from more cases.

In this study, it was clarified that *A. equuli* including subsp. *haemolyticus* and subsp. *equuli* was the primary cause of actinobacillosis in Thoroughbred racehorses in Japan. Each of the 2 subspecies showed different clinical states respectively.

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