Phenotypic and genotypic characterization of Actinobacillus suis sensu stricto isolated from a dairy calf

Michika ISHIHARA1,3), Yuka YAMAZAKI1,4), Ken KATSUDA2) and Hiroya ITO2,5)*

1)Saitama Prefectural Chuo Livestock Hygiene Service Center, Saitama, Japan
2)The National Institute of Animal Health, NARO, Ibaraki, Japan
3)Present address: Saitama Prefectural Kawago Livestock Hygiene Service Center, Saitama, Japan
4)Present address: Saitama Institute of Public Health, Saitama, Japan
5)Present address: The National Institute of Animal Health, NARO, Hokkaido, Japan

NOTE
Bacteriology

ABSTRACT. The species of the genus Actinobacillus have so far been associated with specific animal hosts, and A. suis sensu stricto, an opportunistic pathogen of swine, is rarely isolated from ruminants. We describe here the isolation of A. suis sensu stricto from a newborn calf that died on a dairy farm in Japan. Identification of the isolate was performed by phenotypic and genotypic characterization, with the latter consisting of nucleotide sequence analyses of the 16S rRNA gene plus three housekeeping genes, rpoB, infB and recN.

KEYWORDS: Actinobacillus suis sensu stricto, calf, phenotypic and genotypic analyses

The Gram-negative bacterium Actinobacillus suis, a member of the family Pasteurellaceae, is traditionally thought to be a commensal of the tonsils and upper respiratory tract of pigs [19]. This organism is associated with sporadic and acute cases of septicemia in suckling and weaned pigs, respiratory diseases mainly in grow-finish pigs and acute septicemia in adult animals, with the latter two disease categories being most commonly observed in herds with high health status [9].

The species of the genus Actinobacillus sensu stricto have so far been associated with specific hosts, and the primary host of A. suis is considered to be pigs [3]. However, over the last four decades, there have been a number of reports on the isolation of A. suis-like organisms from a variety of mammals, including an alpaca [12], calves [7], cattle [20], sheep [20] and horses [2, 15]. These “non-porcine” A. suis-like isolates are phenotypically similar to porcine A. suis, but they have not been characterized genotypically. Since misidentification based on phenotypic characterization is a frequent and serious problem among taxa of Pasteurellaceae family members [4], it remains uncertain whether these “non-porcine” isolates, which are identified based on only phenotypic characteristics, are true A. suis or not.

Later, “non-porcine” A. suis-like isolates that are similar to porcine A. suis isolates phenotypically as well as genotypically using the nucleotide sequence analysis of the 16S rRNA gene (16S rrn), which is widely used in the description of bacterial species descriptions, have been described. The hosts of the “non-porcine” A. suis isolates described in these studies were cats [6, 14], a dog [14], a hare [14], and horses [14]. With the exception of horses, the isolates from all these “non-porcine” animals could be identified as true A. suis genotypically, while the 16S rrn sequence analysis revealed that the so-called equine A. suis isolates, which are phenotypically similar to the porcine A. suis isolates, are not true A. suis [14]. The organisms previously classified as equine A. suis have since been reclassified as the Actinobacillus equi subspecies haemolyticus [5].

Most recently, isolates from a rabbit and a hare that resembled porcine A. suis phenotypically and genetically have been classified as true A. suis [17]. In these cases, three housekeeping genes, rpoB, recN and infB, in addition to 16S rrn, were analyzed for the strains, since the use of 16S rrn might lead to false classification in some cases if taken as the gold standard without an additional genetic approach [17].

The present report describes the phenotypic and genotypic characterization of the organisms isolated from a neonatal calf. In addition to 16S rrn, three housekeeping genes—rpoB, infB and recN—were, when necessary, analyzed as additional genotypic approaches for identification of isolates in the present study.

In early April 2018, a four-day-old male calf died on a farm in Japan where 130 milking cows, 5 dairy heifers and 15 dairy calves were being raised. For bacterial isolation, Columbia agar (Difco, Sparks, MD, USA) supplemented with 5% defibrinated...
sheep blood (CASPB) was used, and the plates inoculated from lungs, heart and pleural fluids were incubated at 37°C in the presence of 5% CO₂. After overnight incubation, non-hemolytic and hemolytic colonies, consisting of Gram-negative rods, grew on the CASB inoculated with each sample. The isolates from lung samples were subcultured using the CASB for identification by further biochemical and molecular testing.

An isolate from one of the non-hemolytic colonies, strain 181401, was found to be catalase-negative and oxidase-positive. The other biochemical characteristics of strain 181401 were examined using a biochemical identification kit, ID test HN20-Rapid (Nissui Pharmaceutical, Tokyo, Japan). The seven-digit biochemical profile number generated by the ID test HN-20-Rapid was 7117773, resulting in 79% and 21% relative probability of being *Actinobacillus equuli* and *Actinobacillus suis*, respectively. The biochemical identification kit (Nissui Pharmaceutical) revealed that fermentation of mannitol, which is one of the key characteristics for discriminating *A. equuli* and *A. suis* [3, 5], was positive in strain 181401. As the fermentation of mannitol is typically negative and positive in *A. suis* and *A. equuli* subsp. *equuli*, respectively, and variable in *A. equuli* subsp. *haemolyticus*, and catalase is variable in *A. equuli* subsp. *equuli*, and typically positive in *A. suis* and *A. equuli* subsp. *haemolytica*, respectively [3, 5], strain 181401 did not appear to be *A. suis* or *A. equuli* subsp. *haemolyticus* but rather *A. equuli* subsp. *equuli*. Furthermore, non-hemolytic strain 181401 would rather be *A. equuli* subsp. *equuli* as hemolytic activity can separate *A. suis* and *A. equuli* subsp. *haemolyticus* (hemolytic) from *A. equuli* subsp. *non-hemolyticus* (non-hemolytic) [3, 5]. However, atypical *A. suis* isolates from a snowshoe hare and a rabbit have previously been shown to ferment mannitol and to be non-hemolytic, respectively [17], suggesting that fermentation of mannitol and hemolysis could not be used as key characteristics for identification of the “non-porcine” *A. suis*. Therefore strain 181401 was further characterized by genotypic analyses for definitive identification.

The 16S *rrn* sequence of strain 181401 was first determined as described previously [13]. Homology searches of the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank databases were performed using the BLAST server at the National Center for Biotechnology Information. The 16S *rrn* sequence from strain 181401 showed the highest identity to an atypical *A. suis* field strain R80 isolated from a rabbit [17] (99.6%), followed by an atypical *A. suis* field strain J3-241 isolated from a snowshoe hare [17] (99.4%) (Table 1). Interestingly, strain 181401 showed closer phylogenetic relationships to the type strains of *A. hominis* (99.1%) and *A. equuli* subsp. *haemolyticus* (98.9%) than to the type strain of *A. suis* (98.7%) at the 16S *rrn* sequence level, a finding that has been previously reported for the two “non-porcine” *A. suis* strains R80 and J3-241 (Table 1) [17].

Next, the nucleotide sequences of the housekeeping genes, *rpoB, infB* and *recN*, from strain 181401 were determined as described elsewhere [17, 18] and compared with those deposited in public databases as described above. In addition, the *recN* sequence similarities were used to calculate the whole-genome sequence (WGS) similarities of strain 181401 with the members of the family *Pasteurellaceae* since it has been reported that *recN* sequences could be used to predict whole-genome relatedness between the family [17, 18]. The WGS similarities can be calculated using the following formula:

\[
\text{WGS similarities} = -1.30 + 2.25 \times (\text{sequence identities}) \quad [17, 18, 21]
\]

The *rpoB* sequence from strain 181401 was identical to that of the two “non-porcine” *A. suis* strains R80 and J3-241, while it exhibited ≤98.1% identity to that of other type strains of the genus *Actinobacillus sensu stricto*, including *A. suis* (Table 1). The *infB* sequence of strain 181401 showed the highest identity (94.8%) to *A. suis* strain R80 and also showed ≥94% sequence identity to type strains of *A. equuli* and *A. suis* as well as *A. suis* strain J3-241 (Table 1). The *recN* sequence of strain 181401 was identical to that of the *A. suis* type strain and exhibited very high sequence identity (>99%) to that of the two “non-porcine” *A. suis*, while showing ≤92% identity to type strains of other taxa of the genus *Actinobacillus sensu stricto* as shown in Table 1. The whole-genome similarity value calculated from *recN* sequences was >93% to the type strain of *A. suis*, as well as to the “non-porcine” *A. suis* field strains R80 and J3-241, and ≤77% to the type strains of other taxa of the genus *Actinobacillus sensu stricto* (Table 1). Since the threshold of the whole genome similarity value, calculated from the similarities of *recN*, has been reported to be around 85% for species separation in the family *Pasteurellaceae* [17, 18], strain 181401 could be identified as *A. suis* sensu stricto at the whole-genome similarities level in conjunction with the high similarity values of the *rpoB, infB* and *recN* sequences of strain 181401 to those of the *A. suis* type strain. Accordingly, strain 181401 could be classified as atypical *A. suis* that lacked hemolytic activity but did ferment mannitol. This is supported by the unique phylogenetic relationship to *A. hominis* at the 16S *rrn* level, as seen in the atypical *A. suis* isolates from a rabbit and a snowshoe hare [17].

Nucleotide sequences of the 16S *rrn*, *rpoB*, *infB* and *recN* of strain 181401 have been deposited in the DDBJ/EMBL/GenBank databases as shown in Table 1.

Porcine *A. suis* isolates produce hemolytic/cytotoxic toxins that are genetically and immunologically very similar to ApxI and ApxII toxins of *Actinobacillus pleuropneumoniae* [19]. Structural proteins of ApxI and ApxII toxins are encoded by *apxIA* and *apxIIA* genes, respectively, PCR amplification for the structural genes *apxIA* and *apxIIA* from strain 181401 were performed as described elsewhere [10]. As expected, neither *apxIA* nor *apxIIA* genes were amplified from strain 181401 (data not shown), suggesting that strain 181401 produces no hemolytic ApxI and ApxII toxins.

A representative isolate from the hemolytic colonies that occurred together with the non-hemolytic colonies was named strain 181402. This isolate was found to be catalase- and oxidase-positive. Strain 181402 was identified as *Mannheimia haemolytica*, an important respiratory pathogen of ruminants [16], using the biochemical identification kit (Nissui Pharmaceutical, Tokyo, Japan) described above, 16S *rrn* sequence analysis and species-specific multiplex PCR [1]. The nucleotide sequence of 16S *rrn* of strain 181402, which is identical to that of *M. haemolytica* strain NCTC 9380T (accession number: AF060699), has been deposited in the DDBJ/EMBL/GenBank databases under accession number LC492113. In addition, a slide agglutination test with *M. haemolytica* serovar-specific antisera revealed that strain 181402 shares common antigens with *M. haemolytica* serovar 2 [8]. Taken together,

J. Vet. Med. Sci. 84(5): 624–627, 2022
The results indicate that strain 181402 is **M. haemolytica** serovar 2. To date, 12 serovars have been identified in **M. haemolytica**; serovars 1 and 6 of this organism are most frequently associated with disease in cattle, while serovar 2 is largely considered to be a commensal in the upper respiratory tract of healthy cattle [16]. In contrast, serovar 2 has also been recognized as a causative agent of ovine pneumonia [16] and a peritonitis case in a three-day-old calf in Japan [11]. The ratios of colony numbers of **M. haemolytica** grown on the same CASB plate were approximately 1:1 and 1:0.03 in the right and left lungs, respectively. Accordingly, **M. haemolytica** serovar 2 may be, largely or in part, associated with the death of the newborn calf described in the present study. The contribution of **A. suis** to the cause of death of the calf remains unknown.

We provided a full phenotypic and genotypic characterization of **A. suis**, a bacterial species that is almost exclusively isolated from pigs. Since **M. haemolytica**, an important pathogen of ruminants, was also isolated together with the bovine **A. suis**, further studies are needed to evaluate the pathogenesis of bovine **A. suis** in calves or cattle.

**CONFLICT OF INTEREST.** The authors declare no potential conflicts of interest.

**ACKNOWLEDGMENTS.** The authors would like to thank Dr. Eriko Koike who engaged in the diagnostic necropsy at Saitama Prefectural Chuo Livestock Hygiene Service Center. This study was funded in part by the Ministry of Agriculture, Forestry and Fisheries, Japan and Saitama Prefectural Government, Japan.

**REFERENCES**

1. Alexander, T. W., Cook, S. R., Yanke, L. J., Booker, C. W., Morley, P. S., Read, R. R., Gow, S. P. and McAllister, T. A. 2008. A multiplex polymerase chain reaction assay for the identification of *Mannheimia haemolytica, Mannheimia glucosida* and *Mannheimia ruminalis*. Vet. Microbiol. 130: 165–175. [Medline] [CrossRef]
2. Bada, R., Mittal, K. R. and Higgins, R. 1996. Biochemical and antigenic relationships between porcine and equine isolates of Actinobacillus suis. Vet. Microbiol. 51: 393–396. [Medline] [CrossRef]
3. Christensen, H. and Bisgaard, M. 2004. Revised definition of Actinobacillus sensu stricto isolated from animals. A review with special emphasis on diagnosis. Vet. Microbiol. 99: 13–30. [Medline] [CrossRef]
4. Christensen, H., Kuhnert, P., Busse, H. J., Frederiksen, W. C. and Bisgaard, M. 2007. Proposed minimal standards for the description of genera, species and subspecies of the Pasteurellaceae. Int. J. Syst. Evol. Microbiol. 57: 166–178. [Medline] [CrossRef]
5. Christensen, H., Bisgaard, M. and Olsen, J. E. 2002. Revised definition of Actinobacillus sensu stricto isolated from animals. A review with special emphasis on diagnosis. Vet. Microbiol. 99: 13–30. [Medline] [CrossRef]
6. Daignault, D., Chouinard, L., Møller, K., Ahrens, P., Messier, S. and Higgins, R. 1999. Isolation of Actinobacillus suis from a cat’s lung. Can. Vet. J. 40: 52–53. [Medline]
7. DeBey, B. M., Blanchard, P. C. and Walker, R. L. 1996. Actinobacillus suis-like organisms associated with septicemia in neonatal calves. J. Vet. Diagn. Invest. 8: 248–250. [Medline] [CrossRef]
8. Frank, G. H. and Wessman, G. E. 1978. Rapid plate agglutination procedure for serotyping Pasteurella haemolytica. J. Clin. Microbiol. 7: 142–145. [Medline] [CrossRef]
9. Gottschalk, M. 2012. Actinobacillosis. pp. 653–669. In: Diseases of Swine, 10th ed. (Zimmerman, J. J., Karriker, L. A., Ramirez, A., Schwarz, K. J. and Stevenson, G. W. eds.), Wiley-Blackwell, Oxford.
10. Gram, T., Ahrens, P., Andreasen, M. and Nielsen, J. P. 2000. An Actinobacillus pleuropneumoniae PCR typing system based on the apx and omLA genes—evaluation of isolates from lungs and tonsils of pigs. Vet. Microbiol. 75: 43–57. [Medline] [CrossRef]
11. Harada, N., Takizawa, K., Matsuura, T., Yokosawa, N., Tosaki, K., Katsuda, K., Tanimura, N. and Shibahara, T. 2019. Bovine peritonitis associated with Mannheimia haemolytica serotype 2 in a three-day-old Japanese Black calf. J. Vet. Med. Sci. 81: 143–146. [Medline] [CrossRef]
12. Hill, F. I. and Johnstone, A. C. 1992. Actinobacillosis in an alpaca (Lama pacos). N. Z. Vet. J. 40: 28–30. [Medline] [CrossRef]
13. Ito, H., Takahashi, S., Assai, T., Tamura, Y. and Yamamoto, K. 2018. Isolation and molecular characterization of a urease-negative Actinobacillus pleuropneumoniae mutant. J. Vet. Diagn. Invest. 30: 172–174. [Medline] [CrossRef]
14. Jeannotte, M. E., Slavić, D., Frey, J., Kuhnert, P. and MacInnes, J. I. 2002. Analysis of non-porcine isolates of Actinobacillus suis. Vet. Microbiol. 85: 83–93. [Medline] [CrossRef]
15. Kim, B. H., Phillips, J. E. and Atherton, J. G. 1976. Actinobacillus suis in the horse. Vet. Rec. 98: 239. [Medline] [CrossRef]
16. Klima, C. L., Alexander, T. W., Hendrick, S. and MacAllister, T. A. 2014. Characterization of Mannheimia haemolytica isolated from feedlot cattle that were healthy or treated for bovine respiratory disease. Can. J. Vet. Res. 78: 38–45. [Medline]
17. Kuhnert, P., Korczak, B. M., Christensen, H. and Bisgaard, M. 2007. Emended description of Actinobacillus capsulatus Arseculeratne 1962, 38AL. Int. J. Syst. Evol. Microbiol. 57: 625–632. [Medline] [CrossRef]
18. Kuhnert, P. and Korczak, B. M. 2006. Prediction of whole-genome DNA-DNA similarity, determination of G+C content and phylogenetic analysis within the family Pasteurellaceae by multilocus sequence analysis (MLSA). Microbiology (Reading) 152: 2537–2548. [Medline] [CrossRef]
19. MacInnes, J. I. and Desrosiers, R. 1999. Agents of the “suis-ide diseases” of swine: Actinobacillus suis, Haemophilus parasuis, and Streptococcus suis. Can. J. Vet. Res. 63: 83–89. [Medline]
20. Mohan, K., Muvavarirwa, P. and Pawandiwa, A. 1997. Strains of Actinobacillus spp. from diseases of animals and ostriches in Zimbabwe. Onderstepoort J. Vet. Res. 64: 195–199. [Medline]
21. Zeigler, D. R. 2003. Gene sequences useful for predicting relatedness of whole genomes in bacteria. Int. J. Syst. Evol. Microbiol. 53: 1893–1900. [Medline] [CrossRef]