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NOTE

Bacteriology

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

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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 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 equuli* 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

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sheep blood (CASB) was used, and the plates inoculated from lungs, heart and pleural fluids were incubated at 37°C in the presence of 5% CO_2. 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. *equuli* (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 /equuli* (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:

WGS similarities= $-1.30 + 2.25 \times$ (sequence identities) [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 phylogenic 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. haemolyica* strain NCTC 9380^T (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,

Table 1. Nucleotide sequence similarities of 16S *rrn*, *rpoB*, *infB* and *recN* as well as whole- genome similarities calculated from the similarities of *recN* for isolate 181401

	16S *rrn* [LC492109][f]	*rpoB* [LC492110]	*infB* [LC492111]	*recN* [LC492112]	Whole-genome[g]
1[a]	*Actinobacillus suis* R80 [DQ666561] (99.6%)	*A. suis* R80 [DQ666627] (100%)	*A. suis* R80 [DQ666583] (94.8%)	*A. suis* ATCC 33415^T [DQ410906] (100%)	*A. suis* ATCC 33415^T (95.0%)
		A. suis J3-241 [DQ666620] (100%)			
2[b]	*A. suis* J3-241 [DQ666554] (99.4%)		*A. suis* ATCC 33415^T [AY508857] and [CP009159] (94.7%)	*A. suis* R80 [DQ666605] (99.9%)	*A. suis* R80 (94.8%)
3[c]	*Actinobacillus hominis* NCTC 11529^T [AY362890] and [NR_042866] (99.1%)	*A. suis* ATCC 33415^T [AY362947] and [CP009159] (98.1%)	*Actinobacillus equuli* subsp. *equuli* 19392^T [CP007715] (94.5%)	*A. suis* J324-1 [DQ666598] (99.3%)	*A. suis* J324-1 (93.4%)
		Actinobacillus equuli subsp. *haemolyticus* CCUG 19799^T [AY362935] (98.1%)			
4[d]	*A. equuli* subsp. *haemolyticus* CCUG 19799^T [AF381187] and [NR_114628] (98.9%)		*A. suis* J3-241 [DQ666576] (94.3%)	*Actinobacillus capsulatus* CCUG 12396^T [DQ410908] (92.0%)	*A. capsulatus* CCUG 12396^T (77.0%)
	A. equuli subsp. *equuli* 19392^T [CP007715] (98.9%)		*A. equuli* subsp. *haemolyticus* CCUG 19799^T [AY508858] (94.3%)		
5[e]		*Actinobacillus ureae* CCUG 2139^T [AY362950] (97.6%)		*A. equuli* subsp. *equuli* ATCC 19392^T [DQ410909] (90.8%)	*A. equuli* subsp. *equuli* 19392^T (74.3%)

[a]The best-match type strains of each taxa or "non-porcine" *A. suis* strains (% identity). [b]The next best-match type strains of each taxa or "non-porcine" *A. suis* strains (% identity). [c]The third best-match type strains of each taxa or "non-porcine" *A. suis* strains (% identity). [d]The fourth best-match type strains of each taxa or "non-porcine" *A. suis* strains (% identity). [e]The fifth best-match type strains of each taxa or "non-porcine" *A. suis* strains (% identity). [f]The characters and numbers within square brackets indicate the accession number. [g]The genome similarities calculated from the similarities of *recN* (% identity).

these 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 *A. suis* and *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* sensu stricto from a diseased calf, 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.

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