Yersinia pseudotuberculosis Prevalence and Diversity in Wild Boars in Northeast Germany

Marie Reinhardt,^a Jens Andre Hammerl,^a Katharina Kunz,^a Andrea Barac,^a Karsten Nöckler,^a Stefan Hertwig^a

^aGerman Federal Institute for Risk Assessment, Department of Biological Safety, Berlin, Germany

ABSTRACT In this study, the prevalence of *Yersinia pseudotuberculosis* in wild boars in northeast Germany was determined. For that purpose, the tonsils of 503 wild boars were sampled. The presence of *Y. pseudotuberculosis* was studied by diagnostic PCR. Positive samples were analyzed by cultural detection using a modified cold enrichment protocol. Ten *Y. pseudotuberculosis* isolates were obtained, which were characterized by biotyping, molecular serotyping, and multilocus sequence typing (MLST). In addition, whole-genome sequences and the antimicrobial susceptibility of the isolates were analyzed. *Yersinia pseudotuberculosis* was isolated from male and female animals, most of which were younger than 1 year. A prevalence of 2% (10/503) was determined by cultural detection, while 6.4% (32/503) of the animals were positive by PCR. The isolates belonged to the biotypes 1 and 2 and serotypes O:1a ($n = 7$), O:1b ($n = 2$), and O:4a ($n = 1$). MLST analysis revealed three sequence types, ST9, ST23, and ST42. Except one isolate, all isolates revealed a strong resistance to colistin. The relationship of the isolates was studied by whole-genome sequencing demonstrating that they belonged to four clades, exhibiting five different pulsed-field gel electrophoresis (PFGE) restriction patterns and a diverse composition of virulence genes. Six isolates harbored the virulence plasmid pYV. Besides two isolates, all isolates contained *ail* and *inv* genes and a complete or incomplete high-pathogenicity island (HPI). None of them possessed a gene for the superantigen YPM. The study shows that various *Y. pseudotuberculosis* strains exist in wild boars in northeast Germany, which may pose a risk to humans.

IMPORTANCE *Yersinia pseudotuberculosis* is a foodborne pathogen whose occurrence is poorly understood. One reason for this situation is the difficulty in isolating the species. The methods developed for the isolation of *Yersinia enterocolitica* are not well suited for *Y. pseudotuberculosis*. We therefore designed a protocol which enabled the isolation of *Y. pseudotuberculosis* from a relatively high proportion of PCR-positive wild boar tonsils. The study indicates that wild boars in northeast Germany may carry a variety of *Y. pseudotuberculosis* strains, which differ in terms of their pathogenic potential and other properties. Since wild boars are widely distributed in German forests and even populate cities such as Berlin, they may transmit yersiniae to other animals and crop plants and may thus cause human infections through the consumption of contaminated food. Therefore, the prevalence of *Y. pseudotuberculosis* should be determined also in other animals and regions to learn more about the natural reservoir of this species.

KEYWORDS *Yersinia pseudotuberculosis*, cultural detection, wild boars, whole-genome sequencing, virulence, antimicrobial resistance

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are enteropathogenic species causing diseases termed yersiniosis (1). All pathogenic *Yersinia* strains possess the 70-kb virulence plasmid pYV containing genes for *Yersinia* outer proteins (YOPs) and a type III secretion system (T3SS) (2). Other virulence genes are located on the chromo-

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Address correspondence to Stefan Hertwig, stefan.hertwig@bfr.bund.de.

some of the bacteria (3), but the compositions of virulence genes are different in the three species and may vary between strains of the same species.

Yersiniosis is the third most common bacterial enteritis in Germany and other European countries (4). The clinical manifestation is symptomized by diarrhea, abdominal pain, and fever and occurs mostly in young children (5). The disease is mainly caused by *Y. enterocolitica*, whereas only relatively few cases of yersiniosis have been reported for *Y. pseudotuberculosis*. The reasons for this might be a weak awareness of *Y. pseudotuberculosis* as a gastroenteritis agent, the difficult cultural detection of this species, and different reservoirs of *Y. enterocolitica* and *Y. pseudotuberculosis*. The occurrence of *Y. enterocolitica* is closely associated with pigs. Infections by this species are mainly caused by the consumption of contaminated pork (6–10). *Y. pseudotuberculosis* has a more diverse host spectrum. It is not only a human pathogen but can also infect livestock and pets as well as zoo and wild animals (11–16). For that reason, *Y. pseudotuberculosis* may gain great importance in view of the increasing interest in foods from unconventional production (e.g., game meat and outdoor farming of domestic pigs). In Germany (hunting season 2015 to 2016), the game of wild boars amounted to 8,846 tons. In addition, approximately 2,200 tons of wild boar meat was imported (17). As small quantities of game can be marketed directly by hunters without control by official veterinarians and since pathogenic *Y. enterocolitica* strains have already been isolated from game (18–21), the consumption of wild boar meat and products made of this meat may pose a risk to consumers. Nonetheless, there are currently only few data on the prevalence of *Y. pseudotuberculosis* in game, particularly in Germany. It has been reported that 62.6% of 763 wild boars contained anti-*Yersinia* antibodies, suggesting that *Y. pseudotuberculosis* may exist in German wild boars (22). In Spain, a prevalence of 25% in tonsil samples was determined by PCR (23). A combination of cultivation and PCR analysis was used to determine the prevalence of *Y. pseudotuberculosis* in tonsils, ileocecal lymph nodes, and feces of wild boars in Sweden. Overall, 20% of the animals tested positive for this species (24). In Switzerland, the tonsils and feces of 153 wild boars were investigated (20). The authors detected *Y. pseudotuberculosis* in 20% and 3% of the animals by PCR and culture, respectively. Only tonsils and not feces tested positive. This shows that the isolation of this species is difficult, because a reliable cultural detection method is still lacking. The ISO 10273:2017 (European Committee for Standardization, 2017), which aims at the cultural detection of pathogenic *Y. enterocolitica*, has recently been revised, but the two species are too different to allow the use of this method for the isolation of *Y. pseudotuberculosis*. Thus, the objective of this study was to isolate *Y. pseudotuberculosis* from wild boars by an improved isolation methodology and to characterize the isolates in detail.

RESULTS AND DISCUSSION

Improvement of the protocol for the cultural detection of *Y. pseudotuberculosis*. Although cold enrichment in conjunction with alkaline treatment (20 s in 0.25% or 0.5% potassium hydroxide [KOH] solution) has been used in several prevalence studies on *Y. pseudotuberculosis* (11, 25–27), some authors reported an increased susceptibility of this species to KOH (28–30). We analyzed the KOH susceptibility of seven strains belonging to five serotypes (O:1a, O:1b, O:3, O:4, and O:5), some of which are the most common serotypes in Europe (31). All strains showed very similar and strong susceptibilities to 0.25% KOH. Already after a treatment for 5 s, the viable cell numbers of overnight cultures declined by more than 4 log₁₀ (Fig. 1). We then determined the susceptibility of *Y. pseudotuberculosis* to KOH in spiked wild boar tonsils. The homogenates of tonsils of three wild boars that had tested negative for *Y. pseudotuberculosis* were each inoculated with 10⁶, 10⁷, and 10⁸ CFU/ml. Without KOH treatment, *Y. pseudotuberculosis* could be easily detected on cefsulodin-irgasan-novobiocin (CIN) agar plates. After KOH treatment for 5, 10, or 15 s, *Y. pseudotuberculosis* was detected in only some samples, particularly those spiked with high numbers of bacteria (Table 1). The sensitivity to KOH was not significantly influenced by the biotype, serotype, or multilocus sequence type (MLST) of the strains. According to ISO 10273:2017, bacteria

FIG 1 Effect of 0.25% KOH on the recovery of seven *Y. pseudotuberculosis* strains. Average values of all strains (serotypes O:1a, O:1b, O:3, O:4, and O:5) are shown. The experiments were performed in triplicates; bars indicate standard deviations.

must be treated for 20 ± 5 s with a 1-day-old KOH solution (0.5%). This indicates that KOH treatment is a critical procedure, which was therefore omitted in the prevalence study.

Studies recently performed with 49 *Y. pseudotuberculosis* strains showed that within the first 7 days, the bacteria grew relatively quickly in LB at 1°C (final optical density at 600 nm [OD₆₀₀] of 0.2 to 0.5), whereas thereafter, growth was much slower (32). We examined the growth of 16 *Y. pseudotuberculosis* strains (serotypes O:1 to O:5) of our strain collection in peptone-sorbitol-bile salt (PSB) medium at 4°C for 14 days. Similar *Yersinia* counts were obtained after cultivation for 7 days and at the end of the

TABLE 1 Recovery of *Y. pseudotuberculosis* from wild boar tonsils after KOH (0.25%) treatment^a

Serotype	Initial inoculum (CFU/ml)	KOH treatment ^b			
		None	5 s	10 s	15 s
O:1a	10 ⁶	+	(+)	—	(+)
	10 ⁷	+	—	—	—
	10 ⁸	+	+	+	(+)
O:1b	10 ⁶	+	—	—	—
	10 ⁷	+	(+)	(+)	(+)
	10 ⁸	+	+	(+)	(+)
O:3	10 ⁶	+	—	—	—
	10 ⁷	+	(+)	(+)	(+)
	10 ⁸	+	+	+	—
O:4	10 ⁶	+	—	(+)	(+)
	10 ⁷	+	+	—	—
	10 ⁸	+	+	—	(+)
O:5	10 ⁶	+	—	—	—
	10 ⁷	+	+	—	—
	10 ⁸	+	(+)	+	—

^aThe experiment was performed with three parallel preparations of tonsils from three animals.

^b—, absence of *Yersinia*; (+), *Yersinia* present but detected in only one preparation; +, *Yersinia* present in more than one preparation.

FIG 2 Growth of *Y. pseudotuberculosis* at 4°C with and without preincubation at room temperature. Mean values (CFU/ml) from 16 *Y. pseudotuberculosis* strains (serotypes O:1 to O:5) are shown; bars indicate standard deviations. d, days.

experiment (data not shown). For that reason, all samples taken in the prevalence study were incubated for only 1 week.

PSB medium is better suited for cold enrichment than modified Rappaport medium (MRB) and irgasan-ticarcillin-chlorate (ITC) broth (11, 26, 31). We wanted to find out whether a preincubation step at room temperature prior to cold enrichment enhances the growth of *Y. pseudotuberculosis*, which could facilitate its detection. Figure 2 shows that preincubation in PSB at room temperature for 6 h strongly promoted the growth of *Y. pseudotuberculosis*. After 7 days, the preincubated culture contained approximately three orders of magnitude higher cell numbers than the culture without preincubation. Similar differences were observed after 10 days. It is possible that the preincubation increased the fitness of the bacteria and that it may be beneficial for the isolation of *Y. pseudotuberculosis*. Therefore, this additional step was included in the prevalence study.

Prevalence of *Y. pseudotuberculosis* in wild boars. Five hundred three tonsils were examined by PCR targeting the *wzz* gene. In 32 samples (6.4%), *Y. pseudotuberculosis* was detected by PCR. All positive samples were used for cold enrichment of the bacteria, leading to the recovery of 10 isolates corresponding to a prevalence of 2% ($n = 10$). A prevalence of 2% determined by cultural detection is in good agreement with data (3%) reported from Switzerland (20). In contrast, a much higher prevalence (20%) was obtained by PCR in the Swiss study. The discrepancy between our and the Swiss data may be caused by different protocols that were applied for the isolation of *Y. pseudotuberculosis*, but it is also possible that other factors (e.g., PCR methodology) accounted for it. Seven isolates were obtained from male wild boars, and six isolates came from animals that were younger than 1 year (Table 2). The sex and age of the investigated animals correspond well to all wild boars shot in this region during 2015 and 2016 (in total, 9,433) (33). It is known that with increasing age, a rise of anti-*Yersinia* antibodies is associated with the acquisition of immunity (22), so that older wild boars carry fewer yersiniae than young animals. In addition, the transmission of *Y. pseudotuberculosis* to other wild boars can be restricted by their large-scale habitats and the fact that wild boars live in small sounders.

Typing and antimicrobial resistance of the isolates. Molecular serotyping performed via PCR revealed that seven isolates belonged to serotype O:1a, while two and one belonged to the serotypes O:1b and O:4a, respectively (see Fig. S1 in the supple-

TABLE 2 Prevalence of *Y. pseudotuberculosis* in wild boars in Germany

Age (mo)	No. (%) of animals		No. (%) of positive animals			
			Culture		PCR	
	Female	Male	Female	Male	Female	Male
<12	121	116	2	4	9	8
12–24	95	100	1	2	3	10
>24	39	32	0	1	0	2
Total	255 (100)	248 (100)	3 (0.6)	7 (1.4)	12 (2.5)	20 (4)

mental material). The serotypes O:1a and O:1b are the most common in Europe, Australasia, and North America (34), and serotype O:1 is one of the serotypes that is associated with infections of wild boars, pigs, and humans (19, 35). The isolates were allocated to the biotypes 2 ($n = 7$) and 1 ($n = 3$), but there was no discernible link between biotype and serotype. Similarly, whereas seven isolates were classified as sequence type 42 (ST42), they belonged to different bio-/serotypes (1/O:1a, 2/O:1a, and 2/O:1b) (Table 3). The remaining isolates were assigned to ST9 ($n = 2$) and ST23 ($n = 1$).

The determination of antimicrobial resistance (see Table S1) revealed that the isolates were susceptible to most antimicrobials. One isolate (M129) exhibited a less pronounced sensitivity to azithromycin than the other isolates. Four isolates exhibited growth at trimethoprim concentrations between 1 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$. The most important finding, however, was that except one isolate (M489), all isolates grew in the

TABLE 3 Origin, typing, and virulence genes of *Y. pseudotuberculosis* isolates

Characteristic	M66	M68	M69	M89	M91	M102	M126	M129	M207	M489
Sex of the wild boar	♀	♀	♂	♂	♂	♂	♂	♀	♂	♂
Age group ^a	A	A	A	A	B	A	A	B	B	C
Biotype	1	1	1	2	2	2	2	2	2	2
Serotype	O:1a	O:1a	O:1a	O:1a	O:1a	O:1a	O:1b	O:1b	O:1a	O:4a
MLST	ST42	ST42	ST42	ST9	ST9	ST42	ST42	ST42	ST42	ST23
Gene										
pYV										
yadA ^b	+	+	+	+	+	+	–	–	–	–
virF ^c	+	+	+	+	+	+	–	–	–	–
Chromosome										
inv ^d	+	+	+	+	+	–	+	+	+	+
ail ^d	+	+	+	+	+	–	+	+	+	+
ypmA ^e	–	–	–	–	–	–	–	–	–	–
ypmB ^f	–	–	–	–	–	–	–	–	–	–
ypmC ^g	–	–	–	–	–	–	–	–	–	–
HPI										
IS100 ^h	+	+	+	+	+	–	+	+	+	–
psn ^h	+	+	+	+	–	–	+	+	+	–
yptE ^h	+	+	+	+	–	+	+	+	+	–
irp1 ^h	+	+	+	+	–	+	–	+	+	–
irp2 ^h	+	+	+	+	–	+	+	+	+	–
ybtP ^h	+	+	+	+	+	+	+	+	+	–
ybtQ ^h	+	+	+	+	+	+	+	+	+	–
ybtX ^h	+	+	+	+	+	+	+	+	+	–
ybtS ^h	+	+	+	+	+	+	+	+	+	–
int ^h	+	+	+	+	+	–	+	+	+	–
asnT-int ^h	+	+	+	+	+	–	+	+	+	+

^aA, 0 to 12 months; B, 12 to 24 months; C, >24 months.

^bAF056092.

^cHE805268.

^dNC_006155 (IP 32953).

^eAF335466.

^fAF418982.

^gAF414083.

^hBX936398 (IP 32953).

presence of high concentrations of colistin-sulfate ($>16 \mu\text{g/ml}$). A non-wild-type phenotype for colistin has already been reported for *Y. pseudotuberculosis* (36, 37) but not for *Y. enterocolitica*. The search for antimicrobial resistance genes in the whole-genome sequences of the isolates by ResFinder 3.0 analysis (<https://cge.cbs.dtu.dk/services/ResFinder/>) (38) did not lead to any hits (data not shown). It has been reported that with the exception of the outer membrane, no natural mechanisms of resistance to antimicrobials exist in *Y. pseudotuberculosis* (39). Thus, the insensitivity to colistin may be attributed to mutations in genes responsible for the composition of lipopolysaccharide (LPS). Since colistin is mainly used to treat infections caused by carbapenem-resistant or extended-spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae*, it does not play an important role for *Y. pseudotuberculosis*.

Virulence gene content. The 10 *Y. pseudotuberculosis* isolates were analyzed for the presence of virulence genes located on the chromosome or on plasmid pYV (Table 3). pYV-encoded *Yersinia* outer proteins (Yops) protect the bacteria against the host's immune system and enable the proliferation and spread of the pathogen (40). They have a toxic effect and are secreted into eukaryotic cells by a type III secretion system (T3SS). YadA is a pYV-encoded collagen-binding protein important for autoagglutination, adherence to epithelial cells, and serum resistance, whereas *virF* is a transcriptional activator. The genes *yadA* and *virF* were identified in six *Y. pseudotuberculosis* isolates, and from these, pYV was recovered (data not shown). Moreover, the cultivation of the isolates on Congo red-magnesium oxalate agar (CR-MOX) at 37°C revealed tiny red colonies, suggesting that the calcium response region of pYV was functional. In four isolates, pYV was not detected by PCR or whole-genome sequencing (WGS). In addition, the growth of these isolates on CR-MOX at 37°C indicated that they did not contain pYV. It cannot be ruled out that the four pYV-negative isolates lost their virulence plasmid during cultivation in the laboratory, because pYV is known to be a rather unstable plasmid (41, 42). Strains devoid of pYV are considered to be nonpathogenic, even though pYV-negative *Y. pseudotuberculosis* strains have been isolated from patients (1, 43).

Enteropathogenic yersiniae carry on their chromosomes the genes *ail* (attachment invasion locus) and *inv* (invasin), which promote adherence to and invasion into eukaryotic cells (44). *ail* and *inv* were detected in nine isolates; only one isolate (M102) lacked these genes. In *Y. pseudotuberculosis*, the absence of both genes is rather unusual (45). It is possible that isolate M102 lost the ability to invade the epithelial layer and hence may be a strain with reduced virulence (46–49). *Yersinia pseudotuberculosis* is one of a few Gram-negative bacteria that produce a superantigenic toxin (YPM), whose role in pathogenesis has been discussed (50–52). There are three variants (*ypmA*, *ypmB*, and *ypmC*) of the superantigen gene (53). However, in this study no *ypm* gene was detected in any *Y. pseudotuberculosis* isolate. This finding is in line with observations in studies showing that *ypm* genes are rare in European *Y. pseudotuberculosis* isolates (51), particularly those belonging to the serotypes O:1a and O:1b, which prevailed in this study.

Similar to YPM, the high-pathogenicity island (HPI) is closely associated with symptoms of *Y. pseudotuberculosis* yersiniosis. The HPI encodes proteins that are involved in the biosynthesis, regulation, and transport of the siderophore yersiniabactin (54, 55). For that reason, the HPI has been referred to as an “iron capture island.” A complete high-pathogenicity island (HPI) was detected in six isolates, whereas one isolate (M489) did not possess any HPI-associated gene. This isolate belongs to serotype O:4, which is known to lack an HPI (56). Three isolates possessed a partial HPI in which some genes were absent. There are five genes within the island (*psn*, *irp1*, *irp2*, *ybtP*, and *ybtQ*) that are involved in the yersiniabactin system (54, 57, 58). *Psn* is the outer membrane receptor for the siderophore (59). The genes *irp1* and *irp2* encode high-molecular-weight proteins involved in the nonribosomal synthesis of yersiniabactin (60). *irp2* is a marker of high pathogenicity and is found only in pathogenic strains (61, 62). Isolate M91 lacked the genes *psn*, *irp1*, and *irp2*, while in two isolates (M102 and M126), only

FIG 3 Relationship of the isolates. (A) Clustering determined by whole-genome analysis. Also shown are typing results and the virulence gene content. (B) Numbers of SNPs identified in each isolate.

psn and *irp1*, respectively, were missing. Some other genes of the high-pathogenicity island are considered to be responsible for genetic mobility (56). One of these genes (*int*) encodes an integrase that is similar to the integrase of phage P4 (63). The *int* gene was found in all but two (M102 and M489) of our isolates. These two isolates were the only ones that did not contain the insertion element *IS100*, which is involved in genomic rearrangements (56).

In summary, the analysis of the virulence gene content of the 10 isolates suggests that these isolates differ significantly in terms of pathogenic potential. Only four isolates revealed both the virulence plasmid pYV and important genes (*ail*, *inv*, complete HPI) located on the chromosome. Nevertheless, as the virulence of *Y. pseudotuberculosis* is based on multiple factors, none of the isolates can be regarded as innocuous.

Relationship of the isolated *Y. pseudotuberculosis* strains. To determine the relationship of the *Y. pseudotuberculosis* isolates, initial experiments were performed by pulsed-field gel electrophoresis (PFGE) analysis. Five different restriction patterns were obtained using the enzyme *NotI* (data not shown). Between two and ten restriction fragments differed in the isolates. The PFGE patterns were in good agreement with whole-genome data, on the basis of which the isolates were allocated to four clusters (Fig. 3A). Cluster A comprises five strains, all of them belong to the serotype O:1A and MLST 42. However, the two biotype 2 strains of this cluster exhibited different PFGE restriction patterns than the three biotype 1 strains, even though they did not show significantly more single nucleotide polymorphisms (SNPs) (Fig. 3B). Clusters B and D contain isolates of the same biotype (biotype 2), which, however, have different serotypes and MLSTs (Fig. 3A). SNP analyses confirmed the close relationship of strains within each cluster and the more distant relationship between the clusters. One isolate (M489) differed from all other isolates in its serotype (O:4) and MLST (ST23). This strain is most distantly related to the other strains and was therefore assigned to a single cluster (C).

Conclusions. Our study demonstrates that wild boars may be a source of *Y. pseudotuberculosis* infection in Germany. As far as we know, no cases of yersiniosis caused by the consumption of wild boar meat contaminated with *Y. pseudotuberculosis* have yet been reported. However, considering the large quantities of game meat that are consumed, the rapidly increasing populations of wild boars in Europe, and their spread in the environment, wild boars may play an important role in the epidemiology of *Yersinia* infections. Ten *Y. pseudotuberculosis* strains were isolated from 503 wild boar tonsils, while three times more samples were determined as positive by PCR. The

modified protocol that was applied for the cultural detection of this species may be useful for further studies. The analyses of the isolates revealed that they are rather diverse. This pertains not only to the biotype, serotype, and MLST of the strains but also to their virulence gene content. Thus, several lineages of *Y. pseudotuberculosis* exist in German wild boars, and it is likely that additional groups will be found in the future.

MATERIALS AND METHODS

Sampling and isolation of *Y. pseudotuberculosis*. Tonsils of 503 hunted wild boars were collected between February 2015 and December 2016. The ages and sexes of the animals are summarized in Table 2. The animals were shot in the northeastern part of Brandenburg and in Mecklenburg-Western Pomerania on driven community and individual hunts. After evisceration, the samples were immediately taken with sterile gloves and a scalpel for each animal and placed in sterile closed cups to prevent cross-contamination. The samples were transported to the German Federal Institute for Risk Assessment and stored at 4°C. The samples were homogenized in peptone-sorbitol-bile salt (PSB) broth for 4 min using a Bagmixer 400 VW (10 strokes/s; Interscience, Saint Nom, France) to give a 1:10 dilution. To reduce the number of accompanying bacteria, 10 ml of tissue fluid was further diluted in PSB at a ratio of 1:10 and incubated for 6 h at room temperature, followed by incubation for 7 days at 4°C. Thereafter, genomic DNA was extracted from 500 μ l of homogenate by thermal lysis (95°C for 7 min) and investigated for the presence of *Y. pseudotuberculosis* by PCR using the *wzz* gene encoding the O-antigen chain length determinant as the target (64). Of each positively tested tonsil, 100 μ l of homogenate (dilutions, 10^{-5} , 10^{-6} , and 10^{-7}) were plated on cefsulodin-irgasan-novobiocin (CIN) agar to obtain single colonies (65). The agar plates were incubated at 28°C for 48 h. From each plate, up to 20 presumptive *Y. pseudotuberculosis* colonies exhibiting a small bulls-eye appearance were cultured on lysogeny broth (LB) agar at 28°C for 24 h for further analysis by matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS).

MALDI-TOF MS analysis. Mass spectrometric analyses were performed by use of the MALDI-TOF MS system (Microflex LT, Bruker Daltonics, Billerica, USA). Cell material of 20 suspicious colonies of each sample was investigated. Each colony was analyzed twice. The processing of the sample material for direct transfer was performed by using the standard protocol of Bruker. Measurements were taken with the Bruker real-time classification software (version 3.0) and compared to the MALDI biotyper database. The *Y. pseudotuberculosis* strain IP 32953 (Institut Pasteur, France) was used as a process control.

Bio- and serotyping. Biotyping was applied for both species confirmation and the determination of the biotype. To confirm the species *Y. pseudotuberculosis*, the following biochemical tests were carried out: Voges Proskauer, indole, citrate, sorbitol, sucrose, rhamnose, esculin, and melibiose. The biotype of the isolates was determined by the examination of the metabolism of melibiose and raffinose as well as the metabolic conversion of citrate (66).

Molecular serotyping of isolates was carried out by multiplex PCR using nine primer pairs targeting different regions of the O-antigen cluster of *Y. pseudotuberculosis* (64). Isolates of the BfR strain collection representing different *Y. pseudotuberculosis* serotypes (O:1a, O:1b, O:3, O:4, and O:5) were used as the controls.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed using broth microdilution according to CLSI guidelines (CLSI M07-A10) (67) and EUCAST epidemiological cutoff values (ECOFFs) (<http://www.eucast.org/>).

Bioinformatic analysis of WGS data. DNA regions of interest were derived from WGS data of the isolates and analyzed using different tools of the Lasergene (v14.0) software package (DNASTar, Madison, WI, USA). For *in silico* virulence gene profiling, 15 sequences were selected as previously reported (51, 68) (Table 3). PCR was used to confirm the absence of some genes.

The multilocus sequence types of the *Y. pseudotuberculosis* isolates were determined using the web-based MLST 1.8 analysis pipeline of the Center for Genomic Epidemiology (69).

Phylogenetic relationship (SNP tree) was assessed using the software tool CSI Phylogeny from the Center for Genomic Epidemiology (70).

For bioinformatic analyses, the whole-genome sequences of the following *Y. pseudotuberculosis* genomes (accession numbers) were used: [MNKQ000000000](#) (M66), [MNKR000000000](#) (M68), [MAKS000000000](#) (M69), [NCLA000000000](#) (M89), [MAKT000000000](#) (M90), [MNKT000000000](#) (M102), [MAKU000000000](#) (M126), [NCLF000000000](#) (M489), [NCKY000000000](#) (M207), and [MAKV000000000](#) (M129).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00675-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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