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CASE REPORT

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Isolation and characterization of *Leptospira licerasiae* in Austrian swine — a first-time case report in Europe

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Abstract

Background Leptospiraceae comprise a diverse family of spirochetal bacteria, of which many are involved in infectious diseases of animals and humans. Local leptospiral diversity in domestic animals is often poorly understood. Here we describe the incidental detection of *Leptospira (L.) licerasiae* in an Austrian pig.

Case presentation During an experiment to characterize the pathogenesis of *L. interrogans* serovar Icterohaemorrhagiae in pigs, cultivation of a urine sample from a non-challenged contact pig resulted in growth of a spirochetal bacterium that tested negative for pathogenic *Leptospira* (LipL32 gene). PCR, Sanger sequencing and standard serotyping further confirmed that the recovered isolate was clearly different from the challenge strain *L. interrogans* serovar Icterohaemorrhagiae used in the animal experiment. Whole genome sequencing revealed that the isolate belongs to the species *L. licerasiae,* a tropical member of the Leptospiraceae, with no prior record of detection in Europe.

Conclusions This is the first report describing the occurrence of *L. licerasiae* in Europe. Since *L. licerasiae* is considered to have intermediate pathogenicity, it will be important to follow the geographical distribution of this species and its pathogenic and zoonotic potential in more detail.

Keyword *Leptospira licerasiae*, Pig, Austria, Whole genome sequencing

Background

The genus *Leptospira* currently comprises at least 68 different species, based on their whole genome sequence [1, 2]. These species can be further differentiated according to their pathogenic phenotype into pathogenic, intermediate, and saprophytic species [3, 4]. Thorough bioinformatics analysis enabled further distinction into four clades (P1, P2, S1 and S2) [2]. Besides genetic nomenclature, *Leptospira* are classified according to agglutination with reference antisera in the microscopic agglutination test (MAT) [5]. Thus, 300 different serovars can be distinguished which do, however, not align with the genetic classification system [5, 6]. Pathogenic *Leptospira* play an important role as causative disease agents in man and animals alike and leptospirosis has been acknowledged as the most widespread zoonosis in the world [7]. The

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incidence of the disease is likely to increase due to climate change and its consequences, such as regular floodings [8]. Pathogenic leptospires are often maintained in rodents, from where they spill over into humans and animals [7]. However, animals such as cattle, dogs or pigs may themselves act as maintenance hosts for some serovars [9]. While infections may often be asymptomatic, humans may suffer from an acute febrile illness that can potentially result in multi-organ failure [10]. Leptospirosis in swine is typically associated with reproductive disorders, such as abortion at every stage of pregnancy, decreased number of piglets per litter, the birth of runt piglets, increased weaning-to-oestrus interval, agalactia and the so-called SMEDI syndrome (stillbirth; mummification/maceration; embryonic death; infertility) [7, 9, 11, 12]. According to serological data, leptospirosis is common in swine worldwide, with seropositivity ranging from 13 to 36%, depending on the region [9, 13]. Molecular detection by PCR or even isolation of viable leptospires from clinical cases in swine is rarely successful, at least in Central Europe, a fact that might also be influenced by the submission of inappropriate sample material or quality. Thus, in clinical veterinary practice, leptospirosis is mainly diagnosed based on serological evidence, either by a fourfold rise in MAT-titer between paired sera, or – more often – by a single MAT-titer exceeding a certain threshold (usually 1:100) [9]. Since MAT-reactivity is strongly dependent on the relatedness of the infecting strain against which antibodies are developed and the test strain, poor knowledge of locally prevailing *Leptospira* species strongly hampers diagnostic conclusiveness of MAT, with a high probability of the latter to provide false negative test results, due to an incomplete panel of leptospiral serovars employed for the test [14]. Consequently, it is possible that infection with exotic species or serogroups will be overlooked, posing a potential threat to people exposed to livestock.

Leptospira (L.) licerasiae is a species of intermediate pathogenicity belonging to subclade P2 that was first detected in symptomatic human patients and peridomestic rats in urban, peri-urban, and rural areas of the Iquitos region of the Peruvian Amazon [15]. Serological evidence of infection was found in collared peccaries from the same region [16]. Later, it was also isolated from a Japanese traveler returning from Brazil [17]. Isolation of *L. licerasiae* from environmental samples collected in the Philippines and Malaysia demonstrated that the species is not restricted to South America [18, 19]. Interestingly, *L. licerasiae* was also identified as biopharmaceutical cell culture production contaminant [20]. So far, no reports have demonstrated the identification or isolation of *L. licerasiae* from Europe or animals other than those listed above.

Here, we report the first detection and genetic characterization of *L. licerasiae* isolated from a clinically healthy pig from Austria. Since leptospirosis resulting from *L. licerasiae* infection is easily overlooked by standard molecular and serological diagnostic tools, humans and animals might be exposed by a greater extent to potentially harmful leptospires than previously expected.

Case presentation

In the course of an experimental infection to characterize the pathogenesis of *L. interrogans* serovar Icterohaemorrhagiae that is described in detail elsewhere [21], pig #15 served as one of three contact animals (six months old). Contact animals were co-housed with experimentally infected pigs ($n=3$) after challenge. Control animals ($n=2$) were housed separately throughout the study. All pigs were purchased from the same source (private breeder). All pigs tested negative (MAT titers $\leq$ 1:50) for the *Leptospira* serovars Icterohaemorrhagiae, Bratislava, Canicola, Grippotyphosa, Pomona, Wolffi, Tarassovi, and Hardjo before enrollment into the experiment and contact pigs were co-housed with the experimentally infected animals starting from three hours after challenge until termination of the study. Animals in the challenge group were infected with *L. interrogans* serovar Icterohaemorrhagiae. Blood, urine as well as vaginal swab samples were collected on ten time-points during the study, which was terminated 28 days post challenge by euthanasia of all experimental animals. During necropsy, liver, kidneys, urinary bladder, ovaries, oviducts, and both uterine horns were sampled as described [21]. Real-time PCR specifically targeting the leptospiral *lipL32* gene, cultivation, and MAT against the serovars Icterohaemorrhagiae, Bratislava, Canicola, Grippotyphosa, Pomona, Wolffi, Tarassovi, and Hardjo were performed as described elsewhere [21–23].

A single liquid EMJH-culture from a urine sample collected from pig #15 on day 10 post infection of the challenge group showed spirochetal growth after eight days of incubation. The ample presence of roughly 9–12 µm long, thin, helically coiled, spirochetes with hooked ends was demonstrated in this culture (termed Ages40_isolate) by electron microscopy. The bacteria consisted of a homogeneous protoplasm and two periplasmic flagella (Fig. 1). By PCR, both the urine sample and the culture were negative for the *lipL32* gene, that is associated with pathogenic *Leptospira* [23], but positive by 16S real-time PCR [24] with both probes. No sample from any of the other contact pigs or experimentally infected animals tested positive by either PCR or cultivation. To further identify the spirochetal species, present in, and isolated from the urine sample of pig #15, 16S rRNA PCR and Sanger sequencing was performed (see Supplementary

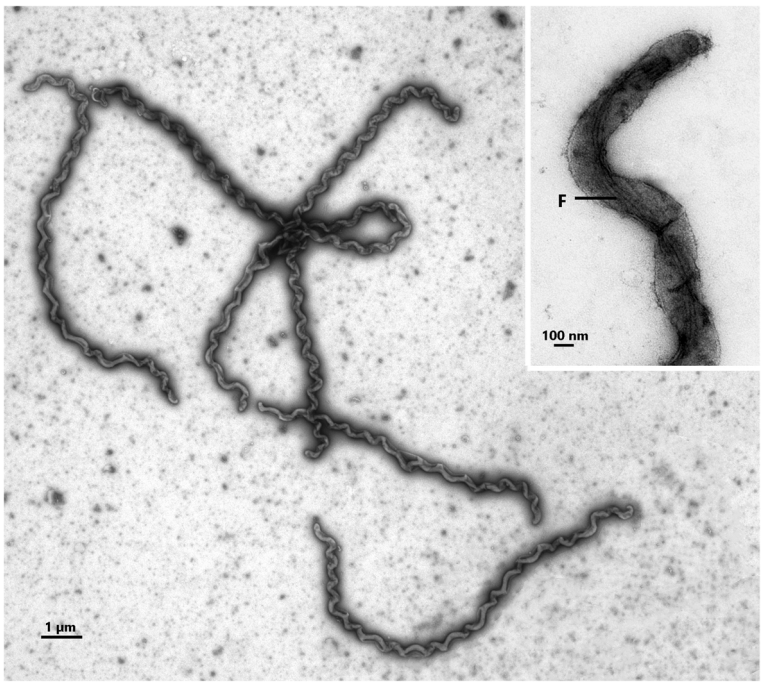

Fig. 1 Negative staining electron microscopy from the AGES40_isolate culture medium showing several tightly coiled leptospires, with two periplasmic flagella (F, insert)

Material). This revealed 620 nucleotide-long 16S rRNA sequences from both the urine sample and the Ages40_ isolate that were 100% identical to several *L. licerasiae* sequences deposited in GenBank. By that it became evident that the Ages40_isolate was not the result of transmission of the challenge strain *L. interrogans* serovar Icterohaemorrhagiae, used to infect pigs in the animal experiment. Upon serotyping, the Ages40_isolate was unreactive in MAT against a panel of 43 anti-*Leptospira* rabbit reference sera and six additional rabbit antisera, including serogroup Iquitos serovar Varillal strain VAR 010 (Supplementary Table 1). However, when serum of pig#15 was tested in MAT against the AGES40_isolate and strain VAR_010, serovar Patoc strain Patoc I and serovar Andamana strain CH11, it reacted with final titres of 1:1280, 1:2560, 1:20 and 1:80, respectively.

To further elucidate the identity of the Ages40_isolate, whole genome sequencing of the isolate was performed, which revealed the presence of a second bacterium that was identified as *Brevundimonas vesicularis*, by bioinformatic analysis (Supplementary Fig. 1) and MALDI-TOF analysis. After the assembly had been cleaned from *Brevundimonas vesicularis* contigs, the final assembly resulted in a 4.28Mbp genome (N50: 138kbp, 82 contigs). We compared the newly sequenced genome to 33 genomes covering different species within the genus *Leptospira* and built a phylogenomic tree based on the core genome of these genomes. The newly sequenced genome clearly clustered with other genomes of the *L. licerasiae* species (Fig. 2). To verify the organisms assignment to *L. licerasae*, we constructed a pangenome and calculated the average nucleotide identity to the same 33 genomes, which confirmed our species assignment (Supplementary Figs. 2 and 3).

Discussion and conclusions

Despite frequent serological evidence, leptospiral infections in livestock are still poorly understood. Awareness about locally prevalent *Leptospira* species and serotypes, their zoonotic potential, the significance of laboratory tests and clinical presentation as well as the prevalence of subclinical infection is presently missing. Here, we report the isolation and characterization of a leptospiral species, *Leptospira licerasiae*, hitherto considered exotic to Europe, from an Austrian pig. This detection happened as a by-product of an experimental trial [21]. No influence of this result on the final conclusions of the previously published animal experiment was conceived, as the finding was limited to an unchallenged contact pig without evidence of clinical or pathological alterations and because any transmission of the challenge strain from the infected pigs was clearly excluded. A shortcoming of the work presented here is the fact that – despite 0.8/0.2 µM filtration followed by

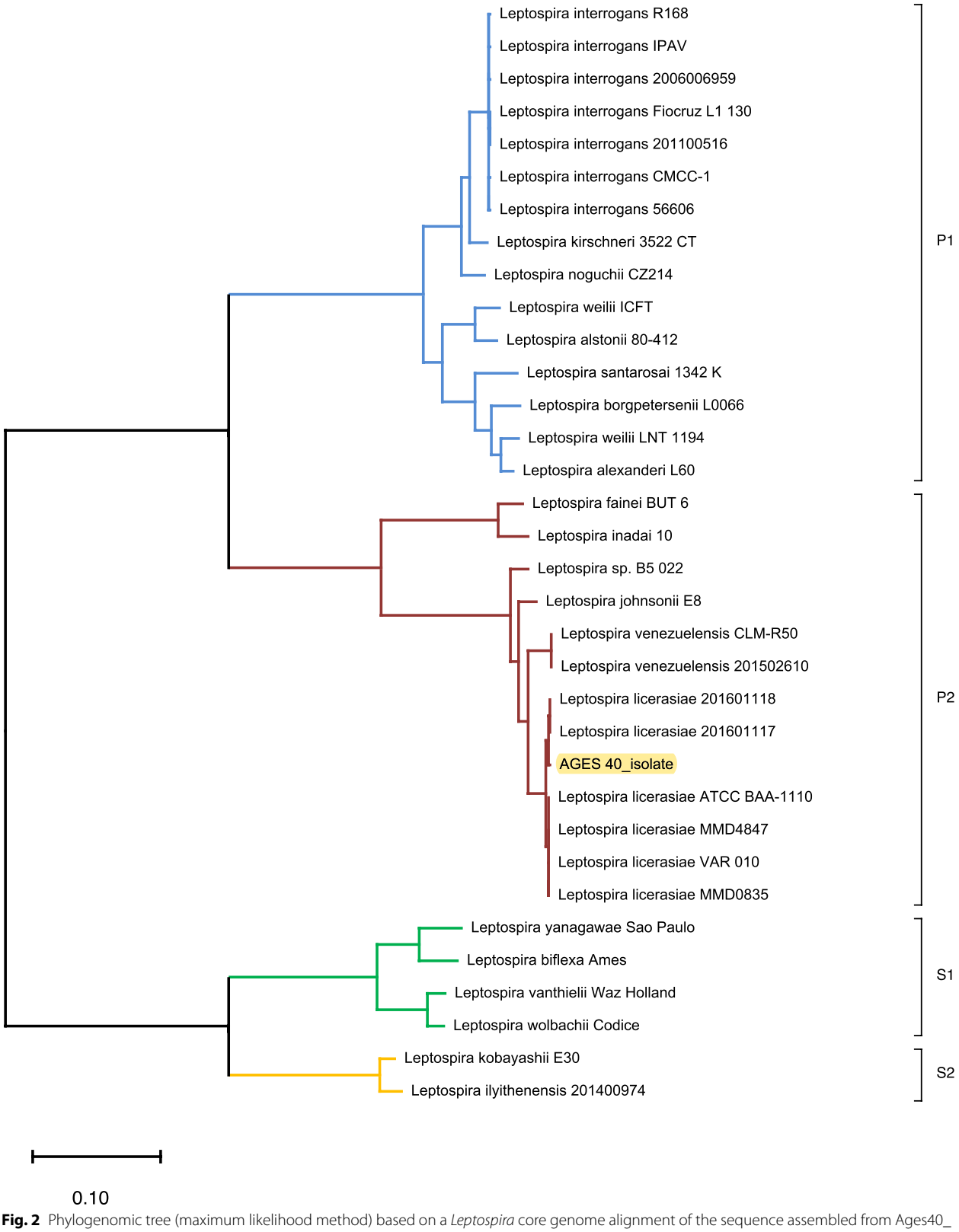

Fig. 2 Phylogenomic tree (maximum likelihood method) based on a *Leptospira* core genome alignment of the sequence assembled from Ages40_isolate (highlighted) and 33 reference genomes from GenBank (see Supplementary Table 3 for metadata). Cluster assignment (P1, P2, S1, S2) is according to Vincent et al., 2019 [2]

plating on solid EMJH-agar – we were not yet able to provide a pure, uncontaminated culture of *L. licerasiae*, that would be necessary for further serological studies with MAT, i.e. to screen both animals such as kept pigs and humans with close contact to them for prior exposure. Thus, so far, infection with *L. licerasiae* has been confirmed in a single pig only. Infection of pig #15 is further supported by the MAT results, where serum from this animal strongly reacted with both the homologous isolate as well as with the related *L. licerasiae* VAR_010 strain. These titers represent a pattern of heterologous reactions that is usually observed in MAT, as antibodies formed during the early infection stage are not strictly specific to the homologous serovar [25, 26].

Since its first description, *L. licerasiae* has been detected by cultural isolation and/or molecular detection in several locations around the world [18, 27, 28], mostly from tropical or subtropical zones. Furthermore, outside of Peru (South America), *L. licerasiae* has so far been described only in studies of the environmental microbiome, but was never isolated from an animal or human host, except in a traveller returning from Brazil [17]. Austria, located in the temperate zone of Europe, is thus a hitherto unknown region for the occurrence of *L. licerasiae* and the fact that it was isolated from a domestic pig makes this finding even more significant. In South America, the potential reservoir animals of this organism are peridomestic rodents and members of the family Tayassuidae, including collared peccaris (*Tayassu tajacu*) [15, 16]. It is currently unclear if European pigs might play a similar role. It is, however, conceivable that *L. licerasiae* infections in Europe have been overlooked so far, as they go undetected by standard molecular and serological diagnostic tools, as was also seen in our study.

Several reports indicate a possible involvement of *L. licerasiae* in human disease [15, 17]. Detection of other members of the "intermediate pathogenic" group of leptospires (now classified as P2) in febrile human patients was documented [29]. Genomic comparisons have also argued that *L. licerasiae* is closer related to pathogenic than to saprophytic *Leptospira*, by sharing more genes with pathogenic strains and having similar metabolic traits, such as Vitamin B12 de novo biosynthesis capabilities [30]. Nevertheless, the *L. licerasiae* lipopolysaccharide composition was found to be substantially different from the one of a pathogenic *Leptospira* species [31].

The identification of *L. licerasiae* in swine from central Europe is at first surprising; however, this might just be a reflection of our patchy knowledge of the global distribution of *Leptospira* species, especially those of intermediate pathogenicity. This picture is likely to change with the ongoing collection of data from environmental studies. It is currently unknown, if *L. licerasiae* is an organism commonly present in swine or in other domestic and wild animals in Europe, let alone if it causes disease in susceptible hosts.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04213-6.

Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.
Supplementary Material 6.
Supplementary Material 7.

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Authors' contributions
AS performed data analysis and wrote the manuscript. AS, CU and RS conceptualized the work. DW and MS performed MAT and bacterial isolation. SR performed electron microscopy. AAA arranged MALDI-TOF and performed PCR and Sanger sequencing. HvdL performed MAT serotyping. DRM and NP performed bioinformatic analysis of NGS data. All authors reviewed the manuscript.

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Availability of data and materials
Next generation sequencing data of the newly described isolate was deposited at DDBJ/ENA/GenBank under the accession JBBBVK000000000. Metadata and accession numbers of the reference sequences used for construction of the phylogenomic tree, the pangenome and the nucleotide identity comparison are listed in Supplementary Table 3.

Declarations

Ethics approval and consent to participate
Ethical approval of the animal experiment, on which the presented work is partly based upon was obtained as described in Steinparzer et al., 2023 [21].

Consent for publication
Not applicable

Competing interests
The authors declare no competing interests.

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