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First Molecular Identification and Phylogeny of a *Babesia* sp. from a Symptomatic Sow (*Sus scrofa* Linnaeus 1758)▽

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Porcine babesiosis is a widespread yet overlooked disease causing economic losses in many regions of the world. To date, the etiological agent of porcine babesiosis has not been molecularly characterized. Here, we provide the first molecular characterization of a piroplasm detected in a symptomatic sow, phylogenetically closely related to the Ungulibabesids. Results pave the way for future molecular epidemiology studies.

Babesiosis, caused by intraerythrocytic parasites of the genus *Babesia*, is one of the most frequently reported infections of free-living and domestic animals. Interest in babesiosis is rising sharply due to its worldwide distribution and public health concerns; indeed, babesiosis is considered an emerging zoonosis of humans (8, 12). Babesiosis is a tick-transmitted disease, with *Ixodidae* ticks being the major vectors. Although swine babesiosis has been reported in the former Soviet Union, Southern Europe, Africa, and China, *Babesia* species affecting pigs have not been studied extensively (2, 10, 15, 16). Two porcine species of *Babesia* are commonly reported, based mainly on the size of the piroplasms detected: *Babesia trautmanni*, transmitted by *Rhipicephalus* ticks and characterized by large piroplasms, and the smaller *B. perroncitoi*, for which the vector still remains unknown (10). Porcine babesiosis is responsible for serious losses and produces antemortem clinical signs that may be similar to those of bovine babesiosis (11). The percentage of parasitized erythrocytes may reach 60%, and abortion associated with fever may appear. Mortality can be significant: 7 out of 56 pigs of a farm died in Italy before therapeutic treatment during an outbreak caused by *Babesia trautmanni*, and in China, a comparable rate was reported during an outbreak of acute babesiosis caused by *B. perroncitoi* (6, 9). However, porcine babesiosis remains an overlooked and neglected disease. Outbreaks of babesiosis in pigs have been very rarely described, with the etiological agents still remaining genetically uninvestigated. Consequently, the DDBJ, EMBL, and GenBank sequence databases lack *Babesia* sequence entries for swine. The lack of molecular data hampers both the development of basic molecular epidemiology studies and the establishment of a role of swine piroplasms, if any, as zoonotic agents.

Here, we report the first molecular identification and phylogeny of a *Babesia* isolate from a symptomatic pig. In summer 2010, EDTA-preserved blood samples and thin blood smears were obtained from a 2.5-year-old pregnant sow reared in a family farm located the region of Anglona (North Sardinia, Italy). The animal showed signs indicative of babesiosis, such as anorexia, depression, lameness, reluctance to move, and high fever, with consequent abortion. Diff-Quick-stained blood smears revealed the presence of babesial inclusions in eryth-

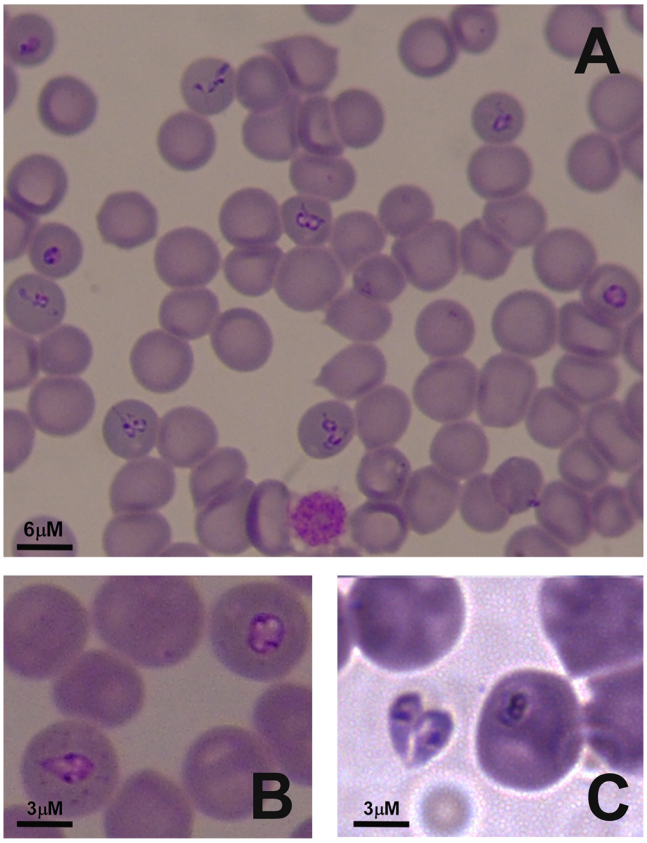

FIG. 1. (A) Thin blood smears revealing the presence of babesial inclusions in erythrocytes. (B and C) Intraerythrocytic (B) and extracellular (C) forms are shown. Pyriform and ring-shaped protozoa were present, as were groups of small intraerythrocytic parasites (A).

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TABLE 1. Taxonomy, GenBank accession numbers, hosts, regions of isolation, and percentages of sequence identity of *Babesia* sp. Suis with the 33 piroplasms used as operational taxonomic units in the phylogenetic analysis

Species	GenBank accession no.	Host	Region	% sequence identity with *Babesia* sp. Suis[a]
Plasmodium falciparum	M19172.1	Human	Africa	64.5
Babesia ovis	AY150058.1	Goat	Spain	94.3
Babesia bovis	AY150059.1	Cattle	Portugal	85.9
Babesia canis canis	AY072926.1	Dog	Croatia	91.8
Babesia canis rossi	DQ111760.1	Dog	South Africa	92.6
Babesia canis vogeli	AY371198.1	Dog	United States	92.3
Babesia equi	AY150064.2	Horse	Spain	88.7
Babesia sp. Kashi2	AY726557.1	Cattle	China	98.0
Babesia occultans	EU376017.1	Antelope	South Africa	98.0
Babesia sp. Kashi1	AY726556.1	Cattle	China	96.3
Babesia orientalis	AY596279.1	Water buffalo	China	97.7
Theileria annulata	M64243.1	Cattle	South Africa	88.5
Theileria mutans	AF078815.1	Cattle	Kenya	88.3
Theileria separata	AY260175.1	Sheep	Germany	88.7
Theileria velifera	AF097993.1	Cattle	Tanzania	89.5
Theileria taurotragi	L19082.1	Antelope	Uganda	88.9
Theileria parva	L02366.1	Cattle	Africa	88.6
Babesia motasi	AY260180.1	Texel sheep	Netherlands	93.8
Babesia bigemina	X59605.1	Cattle	Mexico	93.3
Babesia ovata	AY603400.1	Cattle	China	94.1
Babesia caballi	Z15104.1	Horse	South Africa	94.1
Babesia gibsoni	DQ184507.1	Dog	United States	93.4
Babesia divergens	FJ944826.1	Cattle	France	92.7
Babesia microti	U09833.1	Mouse	United States	86.9
Babesia sp. strain WA1	AY027816.1	Human	United States	87.8
Piroplasmida sp. strain BH1	AF158708.1	Bighorn sheep	United States	87.1
Babesia bovis	M87566.1	Cattle	Australia	84.5
Babesia bovis	L31922.1	Cattle	Mexico	86.0
Babesia odocoilei	AY237638.1	Reindeer	United States	92.8
Babesia sp. strain Dog	AY534602.1	Dog	Spain	86.6
Babesia leo	AF244911.1	Lion	South Africa	86.9
Babesia sp. strain Lion	AF244914	Lion	South Africa	87.1
Cytauxzoon felis	AY679105.1	Cat	United States	87.1
Babesia rodhaini	M87565.1	Small mammals	Africa	86.3

[a] Percentages of identity were evaluated by pairwise alignment and by the sequence identity matrix option of BioEdit (8).

rocytes, with approximately 10% of erythrocytes being parasitized. Smears contained ring-shaped (diameter of 2 to 2.7 μm) and pyriform (1- to 3.3-μm maximum length) protozoa (Fig. 1). Pear-shaped trophozoites occurred either singly (Fig. 1A) or in pairs assembled at their pointed extremities (Fig. 1B). Extracellular forms were also observed (Fig. 1B). The sow recovered after 2 treatments with long-acting oxytetracycline and support therapy (multivitamin complex).

Before treatments, DNA was extracted from blood samples by using the QIAamp DNA minikit (Qiagen, Italy) according to vendor recommendations. Three DNA aliquots obtained from 3 different blood samples were subjected to *Babesia*-specific PCR by using primers described previously (1), with minor modifications. Briefly, primers BT1-F and BT2-R, specifically targeting the entire *Babesia* 18S rRNA gene, were used in a first PCR round. PCR products obtained in the first round were used as the DNA target in 3 heminested PCRs, designed by combining BT1-F with BTH-1R, BT2-R with BT2-F, and BTH-1F with BT2-R. Sequences were obtained by cloning the PCR products obtained from the 3 blood samples into the pCR2.1-TOPO vector (Invitrogen SRL, Milan, Italy) and using an automatic sequencer. At least 3 clones were sequenced for each amplicon. Sequence alignments, obtained with the ClustalW option of BioEdit (7), revealed the presence of an invariable sequence for all the 3 replicates obtained from the 3 distinct DNA samples. Based on sequencing, a consensus sequence was built, containing 1,650 bp of the 18S rRNA gene. Upon performing BLAST analyses, this sequence did not fully match any sequence deposited in the GenBank database but was most closely related (97 to 98% identity with 94 to 100% coverage) to various *Babesia* sequences found in cattle (*Babesia* sp. strain Kashi2), in antelope (*Babesia occultans* and *Babesia* sp. strain Sable Antelope), and in water buffalo (*Babesia orientalis*). This *Babesia* isolate, genetically and geographically distinct from any other previously described species, was tentatively named *Babesia* sp. strain Suis, based on the host in which this pathogen was detected for the first time.

By using the ClustalW option of BioEdit (7), we aligned 1,544 bp of the *Babesia* sp. Suis 18S rRNA gene sequence to 34 sequences (Table 1) of other members of the *Piroplasmida*, representative of the 5 groups identified within this order, as defined previously by Criado-Fornelio and coworkers (1). Phylogenetic analyses using both neighbor joining and maximum parsimony were conducted by use of MEGA, version 4 (14), and yielded coinciding trees where *Babesia* sp. Suis falls into a distinct branch of the Ungulibabesids group, together with *B. orientalis*, *B. occultans*, and *Babesia* sp. Kashi (Fig. 2). The phylogenetic tree is consistent with

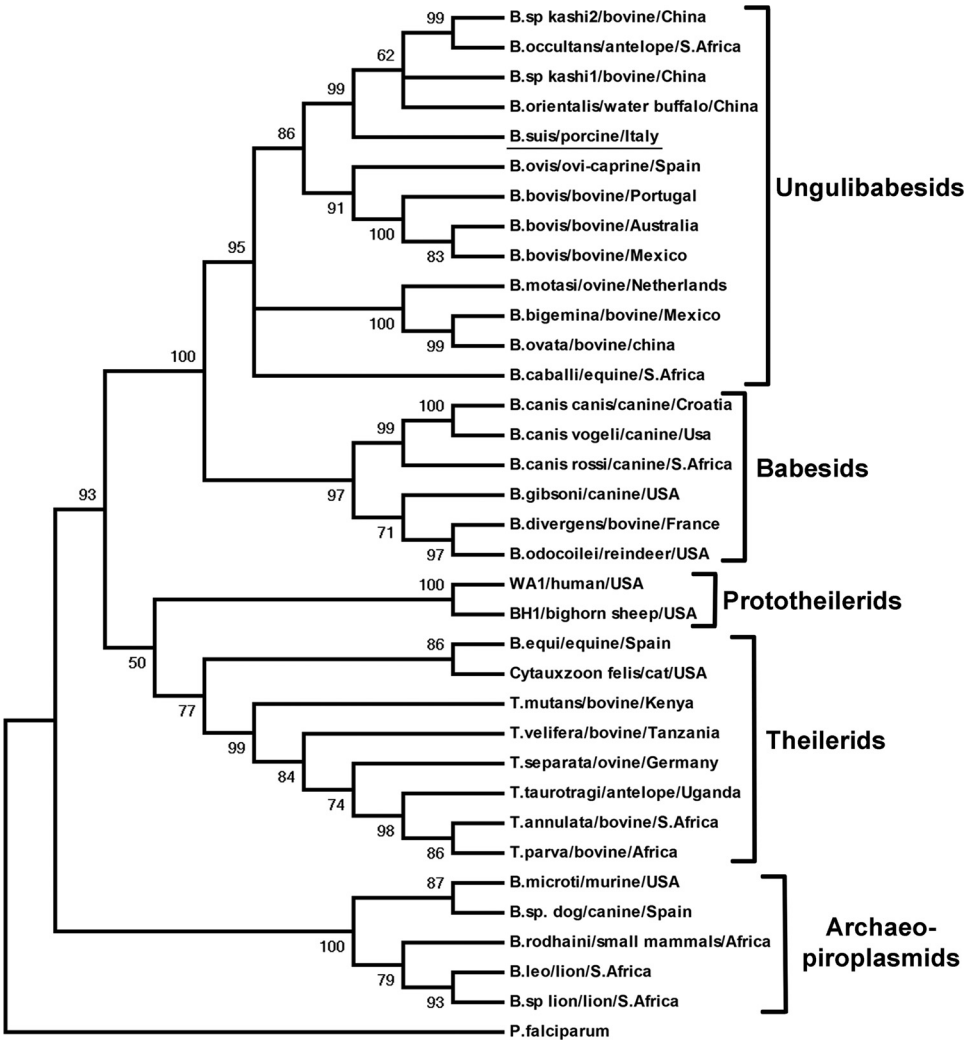

FIG. 2. Coinciding neighbor-joining and maximum parsimony trees showing the evolutionary relationships of *Babesia* sp. Suis and the other 34 taxa used in this study. The Ungulibabesids are spread in 2 branches, one of which has *Babesia* sp. Suis as the most ancestral species. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. All positions containing gaps and missing data were eliminated from the data set (complete-deletion option). There were a total of 1,512 positions in the final data set.

the topology of previously reported analyses based on 18S rRNA gene sequences of piroplasmids (1). Internal branches of the trees were statistically supported by bootstrapping with 1,000 iterations (4).

So far, only two *Babesia* species in pig have been reported, *B. trautmanni* and *B. perroncitoi*, but none of them have been characterized at the molecular level. Moreover, literature on porcine babesiosis is relatively scarce. Notably, babesiosis is of economic importance for pigs, as it can be responsible for serious losses following infection, producing signs not unlike those described for cattle (11).

In this study we report the first molecular characterization of a piroplasm in pig. This species, tentatively named *Babesia* sp. Suis, was detected in a sow from a family farm located in North Sardinia showing symptoms typical of porcine babesiosis, including abortion. Interestingly, an outbreak of porcine babesiosis characterized by a high mortality rate was previously reported in the same area in 1993 (9). However, the lack of molecular data in that previous study and the impossibility of obtaining suitable samples for molecular comparisons render it impossible to verify the homology of *Babesia* sp. Suis with that responsible for the 1993 outbreak. The sizes of the piroplasms described in our study ranged from 2 to 2.7 μm and from 1 to 3.3 μm in ring-shaped (diameter) and pyriform (maximum-length) forms, respectively. These sizes could be indicative of a coinfection with both "small" and "large" piroplasms. However, direct sequencing of amplicons obtained by PCR always resulted in one type of 18S sequence, suggesting the presence of a unique species variable in size. Biometrical polymorphisms within *B. ovis* and *B. canis* were previously reported (5, 13), suggesting that biometrical analysis alone is not sufficient for taxonomical studies of *Babesia* but should always be accompanied by molecular studies. The absence of a vacuole in the ring forms observed would argue for this isolate being *B. trautmanni*. This is also supported by the fact that *Rhipicephalus* is the best-represented tick genus in Sardinia (3). However, the

lack of information on the *B. trautmanni* 18S rRNA gene hampers taxonomical comparisons.

In conclusion, phylogenetic analysis allowed us to place *Babesia* sp. Suis in a distinct ancestral branch of the Ungulibabesids group. The host tropism of its most close relatives (*Babesia* sp. Kashi, *B. occultans*, *Babesia* sp. Sable Antelope, and *B. orientalis*), and their geographical distribution indicate that *Babesia* sp. Suis represents a porcine-specific pathogen. This first molecular characterization paves the way for investigating a possible role of porcine piroplasms as zoonotic agents and establishes a milestone for future molecular epidemiology studies. More data are needed to assess the clinical relevance, the geographical distribution, and the tick vector associated with this *Babesia* sp.

Nucleotide sequence accession number. The partial 18S rRNA nucleotide sequence of *Babesia* sp. Suis was deposited in GenBank under accession number HQ437690.

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