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Emerging human pathogen: Identifying *Spiroplasma ixodetis* as a frequent cause of unlocalised febrile illness

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SUMMARY

Objectives: Febrile illness without clear localisation presents a significant diagnostic challenge due to non-specific symptoms and diverse aetiologies. Spiroplasma ixodetis, an emerging tick-associated pathogen previously linked mainly to congenital cataracts, has not been well characterised in adults. We investigated the prevalence and clinical features of S. ixodetis infection in adults with acute febrile illness without localisation.

Methods: Shotgun metagenomic sequencing identified S. ixodetis in the initial 209 patient cohort and the sequences were used to developed a novel real-time PCR assay targeting the 23S rRNA gene. Initial cohort screening was followed by testing 128 patients from an additionally selected targeted cohort. Positive results were confirmed by sequencing of 16S and 23S rRNA genes.

Results: S. ixodetis DNA was confirmed in 7.2% patients from the initial and in 35.2% patients from the additional cohort (60 in total). All were identified in the period from April to October and 57% reported a recent tick-bite. Clinical presentation was homogenous, characterised by fever, headache, bicytopenia and liver enzyme abnormalities. Outcomes were favourable, with 15% requiring hospitalisation.

Conclusion: This study identifies S. ixodetis as a previously unrecognised cause of adult febrile illness without localisation, bridging the gap between previously published data between tick studies and isolated human case reports.

Introduction

Fever without clinical signs of localisation of infection is a significant diagnostic challenge due to non-specific symptoms, a lack of clear site of infection, and a wide array of potential pathogens.[1] The same is valid for a subset of these illnesses that develop following a recent tick bite.[2] Ticks belonging to the genus *Ixodes* are among the most abundant disease vectors in Europe due to their extensive host range and widespread distribution across the continent.[3] They are known to harbour numerous pathogens.[4] In Slovenia, the most frequent are those causing Lyme borreliosis (LB), tick-borne encephalitis (TBE), and human granulocytic anaplasmosis (GHA).[5] In addition to well-established pathogens, ticks possess highly variable and complex microbiomes composed of commensals and endosymbionts of yet unknown pathogenic potential.[6]

Bacteria from the genus *Spiroplasma*, which belongs to the class *Mollicutes* and the family *Spiroplasmataceae*, are intracellular bacteria with a helical morphology that maintain some of the smallest genomes known for self-replicating organisms.[7] They were first discovered in association with plants, insects, and crustaceans,[7] and they were later found to be endosymbionts inside ticks, where they are transmitted vertically from the females to eggs.[8] The genus currently comprises 38 species, of which *Spiroplasma turonicum*, *Spiroplasma apis*, and *Spiroplasma ixodetis* have been recognised as potential causative agents of human diseases.[9–11] The first

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implication of a spiroplasma as a human pathogen was a case of human transmissible spongiform encephalopathy, but the precise nature of its role remains to be elucidated.[12] In 2015, *S. turonicum* was first documented in a blood culture of a patient with a history of prolonged intermittent fevers and rheumatoid arthritis.[9] In 2018, *S. apis* was linked with unexplained sepsis in a patient with agammaglobulinemia.[10] *Spiroplasma ixodetis* – which was first isolated from *Ixodes pacificus* ticks in the United States,[13] but was later found to be widespread in various *Ixodes* species, as well as in *Dermacentor* spp. and *Rhipicephalus* spp.[14] – has also been associated to human disease.[15–19] However, despite the apparent widespread prevalence of *S. ixodetis* in ticks, a very limited number of cases in humans have been documented to date. Until November 2025, a total of 18 cases of infantile cataracts[11] and two case reports of systemic infection in adults (one immunocompromised patient and one immunocompetent patient) have shown *S. ixodetis* to be the causative agent of the disease.[18] Finally, an organism closely related to *S. ixodetis*, *Spiroplasma* sp. Zurich, was associated with a case of hepatitis in an immunocompromised patient.[16] Although the ability of *S. ixodetis* to cause cataracts in infants is well documented, there are very few data on diseases in adult patients. A larger cohort study and a more robust investigation into the role of ticks as vectors of infection are necessary to improve the understanding of this subject.

The aim of this study was to ascertain the frequency of *S. ixodetis* infection in a well-defined cohort of adult patients having acute febrile illness with no obvious localisation and a negative test result for known tick-borne pathogens and to define the basic clinical findings of the *S. ixodetis*–positive patients. Based on the initial finding of *S. ixodetis* genome fragments in human blood with shotgun metagenomic sequencing (mNGS), we developed a novel, highly sensitive real-time PCR, which allows rapid molecular testing of clinical samples. For this study, positive results were further supported with a well-established 16S and 23S rRNA–targeted NGS protocol.

Materials and methods

Ethics

The study was performed retrospectively on routine leftover samples, from the Institute of Microbiology and Immunology, Faculty of Medicine, and no additional samples were taken for the purpose of the study. All archived samples were used with explicit approval from the National Medical Ethics Committee of the Republic of Slovenia (ethical approvals no. 0120–253/2023–2711-6; amended 23.12.2024 (cohort 1) and 0120–170/2023/4; 9.5.2023 (cohort 2)). The study was conducted according to the Declaration of Helsinki and the Patient Rights Law of the Republic of Slovenia.

Patient cohorts

Shotgun metagenomic sequencing was performed on stored EDTA blood specimens of a cohort of 409 randomly selected adult patients with acute febrile illness (body temperature ≥38 °C; duration ≥2 days) with no obvious localisation of infection. The patients were examined at the Department of Infectious Diseases, University Medical Centre Ljubljana, Slovenia, from 2015 to 2024. The study population consisted of two subgroups: a) 200 patients with a confirmed disease aetiology, previously established using validated molecular tests, and b) 209 patients in whom the cause of disease remained unknown despite rather extensive laboratory testing. A description of 200 patients with confirmed disease aetiologies, as well as a detailed description of the pathogens and the molecular tests used, has been previously published.[20]

Following mNGS detection of *S. ixodetis* DNA in the blood of 15 patients from this initial cohort of 209 patients with unknown aetiology, and realising that these patients had a considerably homogeneous clinical presentation and laboratory findings, and were identified within a limited period from April till October, an additional cohort of 128 adult patients was selected. These patients were diagnosed with acute febrile illness without an obvious localisation of infection during 2021 and 2025, and were negative for *Borrelia* spp., TBE virus, and *Anaplasma phagocytophilum*.

Demographic and basic epidemiologic data, clinical presentation, and laboratory findings were obtained retrospectively from patient charts after determining the presence of *S. ixodetis*.

Shotgun metagenomic sequencing

Shotgun metagenomic sequencing was performed according to the previously published protocol.[20] Samples were individually barcoded and then multiplexed together (up to 70) before sequencing on a NextSeq550 Instrument (Illumina, San Diego, USA) loaded with a NextSeq HighOutput v2.5 cartridge (Illumina, San Diego, USA). Target sequencing yield was at least $5{\times}10^6$ raw reads per barcoded sample. In each run, a positive control (Equid alphaherpesvirus 1, Equine arteritis virus mixture) and a negative control (water) were added. The bioinformatic pipeline consisted of data preparation and quality control, followed by taxonomic classification, *de novo* assembly, and reference mapping. The reference sequence was selected based on the highest score and the percentage identity of the assembled contigs according to BLASTn (see Supplementary).

Development of the 23S rRNA real-time PCR assay

The primers and probe for amplification of a 75 bp fragment of the *S. ixodetis* 23S rRNA gene were designed by aligning *de novo* contigs from mNGS sequenced patients (P06, P11, and P14) and 29 other *Spiroplasma* species from the NBCI database (Supplementary Fig. S1). Table 1 shows the newly designed primers and probe. The real-time PCR was performed in a total volume of 12.5 μL which contained 5 μL of DNA isolate and 7.5 μL of master mix. The master mix was composed of 1× TaqMan®Fast Virus 1-Step Mix (ThermoFischer Scientific, Waltham, USA), 0.5 μM primer (each), 0.25 μM probe (all TIBMOLBIOL Berlin, Germany), and molecular biology grade water (Qiagen, Hilden, Germay). Cycling conditions were as follows: 50 °C for 5 min and 95 °C for 30 sec, followed by 40 cycles of 95 °C for 3 sec and 60 °C for 30 sec. PCR was performed on a QuantStudio™7 Pro instrument (Thermo Fischer Scientific, Waltham, USA).

The primers and probe were bioinformatically checked for human genome homology using BLAST and then tested for analytical specificity using EDTA blood samples from healthy donors, as well as clinical samples containing DNA from the following microorganisms: *Anaplasma phagocytophilum*, *Bartonella henselae*, *Brucella* spp., *Rickettsia* spp., *Coxiella burnetii*, *Mycoplasma pneumoniae*, *Mycoplasma hominis*, *Mycoplasma genitalium*, *Ureaplasma urealyticum* and *Ureaplasma parvum* and cell lines contaminated with several mollicute species (see Supplementary). To assess the analytical properties of the new 23S real-time PCR (rtPCR) further, a gBlock quantitative standard (Integrated DNA Technologies, Coralville, USA) was designed and spiked into a healthy donor EDTA

Table 1
Newly designed 23S rRNA real-time PCR primers and probe.

Name	5′-mod	Sequence	3′-mod
S.ixo_23S-F	None	TGCCTACACAACCCTCTTTGTA	None
S.ixo_23S-R	None	TGGAATGACGGAGAAGGCTAAA	None
S.ixo_23S-Pr	FAM	AGCCCAGYACTACCAAYCACTGGGACA	BHQ1

Y = degenerated base (C or T)

blood, producing a logarithmic dilution series ranging from 10^6 to 0.1 target copies/μL of nucleic acid isolate. PCR efficiency, limit of detection (LoD) and intra-/inter-assay reproducibility were tested.

16S rRNA metabarcoding

Metabarcoding (a targeted high throughput sequencing approach amplifying the full-length 16S rRNA gene) was performed with EasySeq™ Full-length 16S Library Prep Kit (NimaGen, Nijmegen, Netherlands) that allows for the amplification of the entire 16S rRNA gene (covering variable regions V1 – V9) with indexing and adaptor addition in a single PCR reaction. Briefly, 12 μL of mastermix containing Full-length 16S Probe Panel, Probe Dilution Buffer and HiFi Polymerase was used to resuspend dehydrated barcodes in the EasySeq™ Barcode Plate (all (NimaGen, Nijmegen, Netherlands). After adding 8 μL of sample DNA and running the manufacturer's PCR programme, the barcoded PCR products (up to 94 samples with a negative (water) and positive (*Corynebacterium ulcerans*) control) were polled together and purified with AMPure XP beads (Beckman Coulter, Brea, CA, USA) in a 0.6:1 bead to pool ratio. Following quantification using Qubit dsDNA High Sensitivity assay on a Qubit3 fluorimeter (ThermoFisher Scientific, Waltham, USA) and normalisation to 200fmol, library preparation was done with a Ligation Sequencing Kit V14 (Oxford Nanopore Technologies, Oxford, UK) and sequenced on a GridION instrument loaded with a MinION Flow Cell (Oxford Nanopore Technologies, Oxford, UK) for 48 h or until sequencing plateau was reached (see Supplementary).

Development of 23S rRNA–targeted sequencing

As an additional test, we designed a new primer pair (Sixo23S_seqF: 5′ GGT CAA TCG GCA TGA AGG ATT C-3′ and Sixo23S_seqR: 5′ AGC CGG CGA GTT ACG ATT A-3′) to amplify a 743-base fragment of *S. ixodetis* 23S rRNA located downstream of the real-time PCR. The PCR was performed as follows: 50 °C for 30 min and 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 sec, 57 °C for 30 sec, and 72 °C for 60 sec, and a final elongation at 72 °C for 5 min. The master mix was composed of 1× PrimeScript Buffer (Takara Bio, Kusatsu, Japan), 1× PrimeScript 1 step Enzyme mix (Takara Bio, Kusatsu, Japan), 0.35 μM each primer, molecular biology grade water up to 20 μL, and, finally, 5 μL of template DNA. PCR products were cleaned using AMPure XP beads (Beckman Coulter, Brea, CA, USA) in a 1:1 ratio and used for library preparation with the Native Barcoding v14 Kit (Oxford Nanopore Technologies, Oxford, UK) following the manufacturer's instructions. Sequencing was performed on a P2 Solo instrument (Oxford Nanopore Technologies, Oxford, UK) loaded with a PromethION flow cell (Oxford Nanopore Technologies, Oxford, UK) for 24 h until a sequencing plateau was achieved (see Supplementary).

Results

This study aimed to identify the causative agent(s) of an undifferentiated febrile illnesses without localisation, using several different unbiased metagenomic approaches. A summary of the events leading to the discovery of *S. ixodetis* being a relatively frequent and sole pathogen in patients with unexplained febrile illness is shown in Fig. 1.

Identification of S. ixodetis *with shotgun sequencing*

mNGS was performed on a total of 409 patients, and *S. ixodetis* reads were only found in 15 of the 209 patients (7.2%) in whom the aetiology of the disease was unknown. No other known viral, bacterial, parasitic or fungal pathogens were identified in these samples (Fig. 1). The average number of raw reads for these samples was 7,922,530 (minimum–maximum: 4,568,337–11,335,520). Following the depletion of the host reads by mapping them to the human reference genome (GRCh38), an average of 1,736 unique *S. ixodetis* reads (min–max: 3–16,560) were found in 14/15 samples. However, in one sample, although no unique *S. ixodetis* reads were found, 540 *Spiroplasma* genus reads were present. *De novo* contig construction yielded 43 contigs belonging to *S. ixodetis* (on average three per sample; min–max: 1–6), with an average length of 436 base pairs (bp; min–max: 225–1,975). Thirty-seven of these corresponded to 23S rRNA and six to 16S rRNA, with average similarities of 98.5% and 99.6%, respectively (Table 2).

Reads mapping to the *S. ixodetis* Y32 reference genome (NZ_CP127039.1) showed distinct spikes in coverage at genomic positions corresponding to 16S and 23S rRNA. Although most of the reads align with the 16S rRNA and 23SrRNA, other regions of the genome are also covered in scattered smaller pieces (Fig. 2). The average recovery rate at 10× coverage was 58.6% (883/1,508 nt) for 16S rRNA and 22.6% (673/2,985 nt) for 23S rRNA in operon 1. In operon 2, the average recovery rate (10×) was 66.1% (996/1,508 nt) for 16S rRNA and 96.8% (2,842/2,935 nt) for 23S rRNA. The average recovery rate for the rest of the genome was 0.62% (12,572/2,031,352 nt).

Development of the 23S rRNA real-time PCR assay

Based on the mNGS results, we developed a *S. ixodetis*–specific rtPCR to amplify 75 bp of the 23S rRNA gene. Bioinformatic analysis reveal no significant homology with human genome and analytical specificity testing yielded no positive results with any of the organisms, mollicute-contaminated cell lines, or healthy human blood samples. The PCR efficiency was found to be 101.5% (slope −3.286) with an R^2 value of 0.995. Intra-assay reproducibility showed no discrepant results, with an average coefficient of variation (CV) of 1.6%. Similarly, inter-assay reproducibility also showed no discrepant results, with an average CV of 1.9% (Supplementary Table S1). Probit analysis revealed LoD_{95} to be 0.772 copies/μL (95% CI: 0.635 – 1.16).

Therefore, we tested clinical samples from 337 patients (209 from cohort 1 and 128 from cohort 2) (Fig. 1), and a total of 60/337 (17.8%) were found to be positive. In the first cohort, only the same 15 patients were positive with the developed rtPCR, while in the second cohort, 45 patients (35.2%) were positive. The performance of the rtPCR was similar when testing whole blood or plasma in terms of qualitative results. However, there was a significant difference in the Ct values. The average Ct value for the 60 positive whole blood samples was 33.03 ± 2.5 (min = 27.2; max = 37.2), whereas the average Ct value for the plasma samples was 29.1 ± 2.7 (min = 24.0; max = 34.7). A paired two-tailed t-test showed a significant difference between conditions ($t(59) = 17.69$, $p < 0.0001$), with a mean paired difference (plasma – cell fraction) of −3.88 ± 1.70 and a 95% confidence interval of −4.32 to −3.44.

Sequencing of 16S and 23S rRNA and phylogenetic analysis

Full-length 16S rRNA and 23S rRNA–targeted sequencing were performed on all rtPCR-positive samples (Fig. 1). Overall, 58/60 rtPCR-positive patients (96.7%) were confirmed by either 16S or 23S rRNA. Two rtPCR-positive samples were not confirmed by either sequencing approach. These samples had the lowest *S. ixodetis* DNA concentrations, with Ct values of 33.0 and 33.3, respectively, however, the two unconfirmed patients also had *S. ixodetis* 16S rRNA reads detected in the samples, but the number of reads was below the established threshold (Supplementary Table S2).

The 16S rRNA sequences corresponding to *S. ixodetis* were detected in 47/60 samples (78.3%). In addition, 16S rRNA metabarcoding was also performed on 83 rtPCR-negative samples (Fig. 1), and none of the samples yielded any sequences corresponding to *S.*

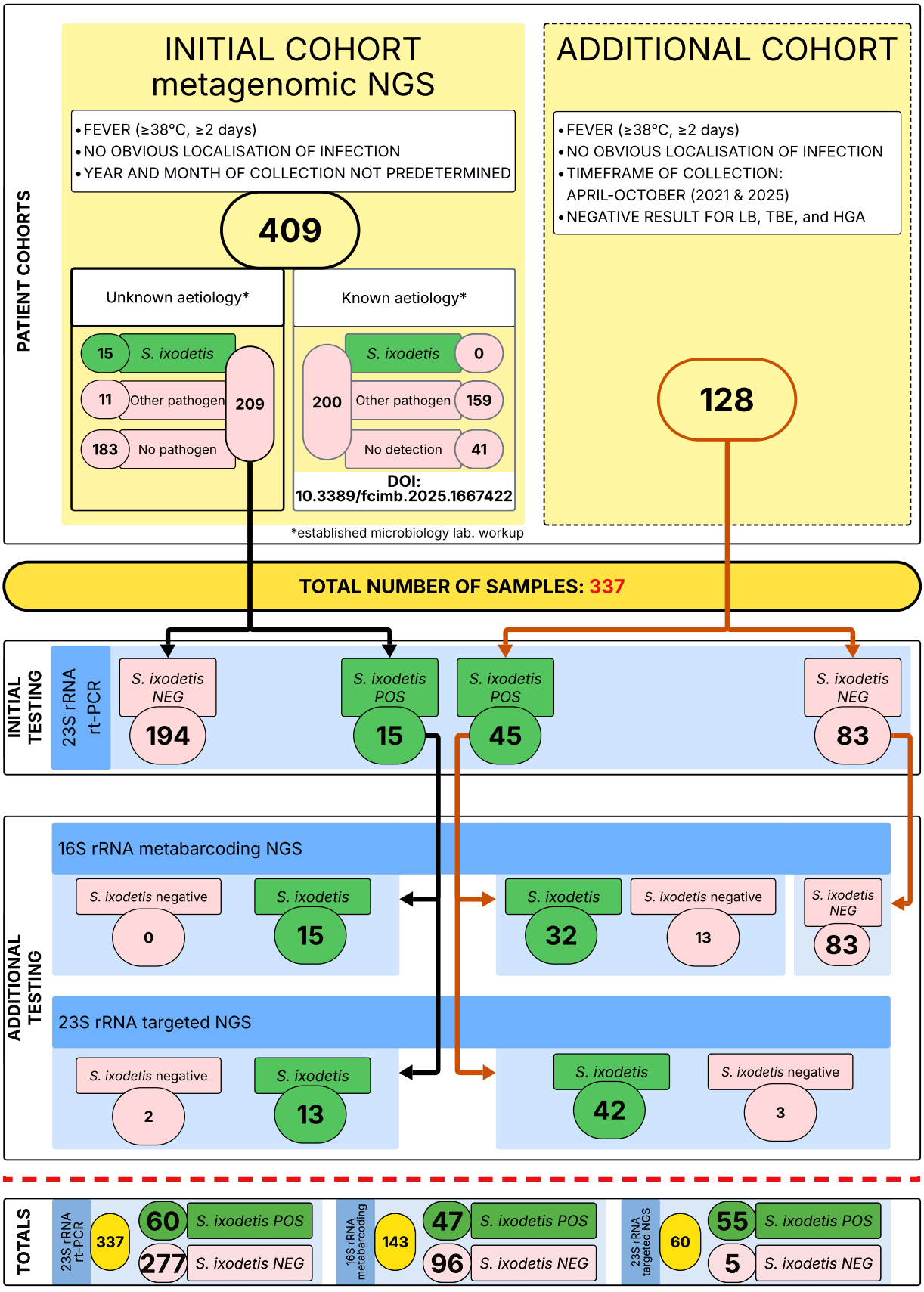

Fig. 1. Detailed summary of investigation leading to discovery of *S. ixodetis* in febrile patients. LB – Lyme borreliosis, TBE – tick-borne encephalitis, HGA – human granulocytic anaplasmosis.

Table 2
Detailed mNGS results for the 15 samples containing *S. ixodetis* reads.

Sample ID	mNGS raw reads	*S. ixodetis* unique reads (> 120 bases)	*S. ixodetis* reads (per 10^7 raw reads)	*de novo* contigs no.	16S rRNA contigs† no. (length) [% similarity]	23S rRNA contigs† no. (length) [% similarity]	Non *S. ixodetis* contig no.
P01	11,335,520	589	518	6		5 (494, 493, 451, 365, 314) [99.4, 99.2, 97.4, 96.6, 98.2]	1*
P02	6,086,251	315	516	4		4 (390, 379, 304, 295) [99.5, 98.9, 100, 97.2]	0
P03	7,000,192	20	29	2		2 (391, 229) [100, 97.7]	0
P04	7,517,364	3	4	3	2 (336, 316) [100, 100]	1 (324) [98.9]	0
P05	7,937,723	27	32	2		2 (414, 272) [100, 98.6]	0
P06	10,869,023	2,341	2,154	4		4 (656, 656, 303, 301) [97.9, 97.9, 98.3, 98.6]	0
P07	6,883,197	116	169	1		1 (284) [97.4]	0
P08	8,343,451	54	65	2		2 (485, 316) [99.2, 98.1]	0
P09	5,103,864	0	0	2	2 (382, 296) [99.5, 99.5]	0	0
P10	8,003,348	1,767	2,208	4		3 (457, 438, 409) [99.7, 98.1, 98.7]	1**
P11	10,954,715	336	307	2		2 (525, 348) [99.6, 98.0]	0
P12	8,076,706	16,560	20,503	6	2 (345, 225) [98.5, 100]	4 (1,975, 539, 523, 486) [98.6, 98.7, 99.8, 97.8]	0
P13	9,456,204	11	12	2		2 (542, 537) [99.2, 99.2]	0
P14	6,702,048	2,133	3,181	3		3 (577, 587, 267) [97.5, 97.7, 98.4]	0
P15	4,568,337	27	57	2		2 (330, 314) [97.5, 97.5]	0

†*S. ixodetis*
* 319 bp long contig corresponding to *E. coli* GMP/IMP nucleotidase;
** 543 bp long contig corresponding to *C. acnes* PTS Galactitol transporter

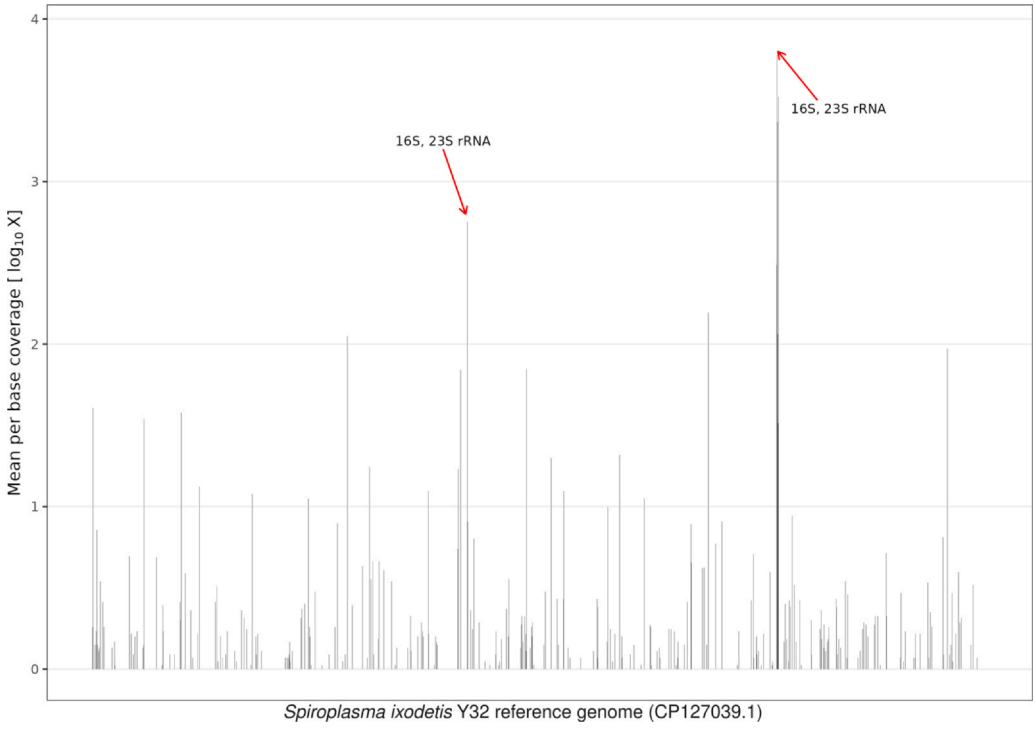

Fig. 2. Genome coverage plot of 14 *S. ixodetis*–positive patients based on mNGS reads mapping to the *S. ixodetis* Y32 reference genome (NZ_CP127039.1). The *x*-axis shows the genomic positions of the reference genome, and the *y*-axis shows the mean coverage on a $\log_{10}$ scale. Red arrows mark locations corresponding to the *S. ixodetis* 16S and 23S rRNA operons.

ixodetis. The comparison between the developed rtPCR and established 16S rRNA metabarcoding demonstrated strong overall agreement of 90.9% (95% CI: 85.1–94.6%) (Supplementary Table S3). Among the 47 positive patients, 40 had *S. ixodetis* reads detected in all four bioinformatic pipelines (4/4), and seven had reads detected in 3/4 pipelines. Phylogenetic analysis revealed a distinct and well-

supported *S. ixodetis* clade. The 16S rRNA gene sequences obtained from the patients in this study clustered together with those previously published from paediatric cases in France and Germany. Above that, sequences showed high similarity to the sequence obtained from ticks collected in the same geographic area in June 2025 (Fig. 3A).

For 23S rRNA sequencing, we successfully amplified the targeted region in 55/60 (91.7%) rtPCR patients. Eleven patients were confirmed exclusively by 23S rRNA–targeted sequencing. In these cases, *S. ixodetis* 16S rRNA reads were detected but remained below the predefined threshold. The average Ct value for these 11 samples was 31.3 (range: 29.5–34.7). On average, 46% of the bacterial reads corresponded directly to the species *S. ixodetis*. The majority of the remaining reads were identified as unclassified *Spiroplasma* species, closely related to *S. ixodetis*, with only a few corresponding to other bacterial species, such as *Staphylococcus hominis* and *Staphylococcus epidermidis*. The phylogenetic analysis of the 23S rRNA gene showed a similar monophyletic *S. ixodetis* cluster, but with slightly more branches, indicating the less conserved nature of 23S rRNA (Fig. 3B).

Clinical presentation

Demographic and basic epidemiologic data, clinical presentation, and laboratory findings were obtained retrospectively from patient charts. Of the 60 patients with *S. ixodetis* DNA detected in their blood, no other pathogens were identified. Of the patients, 29 (48.9%) were women and 31 (51.7%) were men. Their ages ranged from 19 to 73 years, with a median age of 45.5 years. A tick bite during tick activity season was reported by 42/60 patients (70%) and among them 34/60 (57%) specifically recalled a tick bite from one to three weeks before symptoms onset. The clinical presentation was rather homogeneous. All patients developed an acute illness with a fever ≥38°C and a duration ≥2 days (inclusion criteria). Headache was reported by 57 patients (95%), nausea and/or vomiting by 17 patients (28.3%), and myalgia and/or arthralgia by 23 patients (38.3%). All but three patients (95%) had an elevated C-reactive protein (CRP) concentration (> 10 mg/L). Leukopenia and/or thrombocytopenia were observed in 57 patients (95%), with the lowest leucocyte concentration being 1.5×10^9/L and the lowest platelet concentration 51×10^9/L. Elevated activity of at least one liver enzyme (> 1× over Upper Normal Limit − UNL) was observed in 58/58 (100%) patients that had these test results available. Despite extensive microbiologic testing prior to this study, no cause of the illness was identified.

Nine patients (15%) were hospitalised for 2–9 (median 4) days. Twenty-five patients (42%) received empirical doxycycline for suspected HGA, which was however not confirmed by molecular testing. Thirteen patients were followed up for up to 2 months after the onset of symptoms. In all these patients (treated or untreated), the outcome was favourable, with normalisation or pronounced improvement of laboratory findings at the final visit. The remaining 47 patients were not scheduled for follow-up but were advised to return if they experienced persistent or worsening symptoms; none of them did return, suggesting a self-limiting course of disease.

Discussion

This study originated as an ongoing effort to improve undifferentiated febrile illness diagnostics in Slovenia. The initial metagenomic sequencing approach included 409 patients and aimed to identify both established and unexpected pathogens associated with febrile illness with no obvious localisation of infection. In the subgroup of 200 patients with a known aetiology according to currently used microbiologic diagnostics, mNGS revealed a matching aetiology in 159 cases (79.5%).[20] Among the 209 patients with unknow aetiology, a potential causative agent was identified in 26 patients, including 15 individuals (7.2%) with *S. ixodetis* DNA detected in their blood; in contrast, no *S. ixodetis* was detected in the first subgroup of patients with a known aetiology.

There is quite a lot of information on the presence of *S. ixodetis* in ticks, but very few reports of the disease caused by *S. ixodetis* in humans. A PubMed literature search, performed at the end of November 2025, revealed 18 cases of infantile cataracts in studies from different countries[11] and two case reports of systemic infection in adults.[18] These cases were established based on the current molecular diagnostic approach to detect spiroplasmosis in humans (i.e. detection of bacterial DNA with broad-range 16S rRNA PCR, followed by Sanger sequencing),[11,18] whereas in ticks, other genes (rpoB, ITS) were also targeted by real-time PCR.[21] Based on higher recovery rates of the 23S rRNA gene and higher consensus sequence quality compared to 16S rRNA or other genes from our sequenced patients, we chose this site for the development of a rtPCR assay. With the developed rtPCR, we confirmed *S. ixodetis* DNA in all 15 original patients and detected 45 additional patients (35.2%) from the second cohort of adult patients with unlocalised febrile illness. The analytical and clinical specificity of the rtPCR showed no cross-reactivity with related bacteria. Moreover, all mNGS-negative patients were also confirmed negative by the newly developed rtPCR, strengthening confidence in the novel assay. We demonstrated strong agreement between the developed rtPCR and sequencing because 96.7% (58/60) of rtPCR-positive patients were confirmed by either 16S or 23S rRNA sequencing. The discrepancy between 16S and 23S rRNA sequencing results is most likely explained by methodological differences between the two approaches. Broad-range 16S rRNA sequencing relies on universally conserved primers that generate a long amplicon (~1500 bp) and reflect the overall microbial composition of the sample, resulting in a relatively low level of target-specific enrichment. In contrast, the 23S rRNA–targeted sequencing approach employs primers targeting *S. ixodetis* directly and amplifies a shorter region (~750 bp), which likely facilitates the positive results also in samples with low bacterial loads. This is supported by the high Ct values observed in samples confirmed only by 23S rRNA sequencing. Similarly, the failure of 23S rRNA amplification in samples that were weakly positive by 16S rRNA sequencing can be attributed to low DNA concentrations near the detection limit of both methods. Although false-positive rtPCR results cannot be fully excluded for the two unconfirmed cases, this interpretation appears unlikely given the presence of *S. ixodetis*–specific 16S rRNA reads and the uniformity of clinical presentation across confirmed patients. Together, these findings suggest that the developed rtPCR assay may exhibit higher sensitivity than sequencing-based confirmation in samples with very low bacterial loads.

The phylogenetic analysis of the 16S rRNA gene showed clustering of sequences from febrile adult patients from this study together with sequences of infantile cataract cases from France and Germany.[11] Furthermore, all 60 patients exhibited remarkably homogeneous clinical and laboratory presentation, which provided further support to sequencing results. All our patients with *S. ixodetis* detected in blood were adults, none were immunocompromised, all had an acute febrile illness with the onset within the tick-activity period (from April to October), all had negative laboratory test results for LB, TBE, and HGA, and none presented with ocular involvement as seen in infants.[11] We demonstrated a higher concentration of *S. ixodetis* DNA in plasma compared to whole blood. This finding suggests that either *S. ixodetis* circulates freely in the blood, unattached to cells, or that the site of infection might be elsewhere, with only cell-free DNA fragments circulating in the blood. The first possibility is supported by the findings of Aquilino et al., who managed to isolate *S. turonicum* in a blood culture, albeit with several modifications.[9] Conversely, Mueller et al. showed structures consistent with *Spiroplasma* sp. cells in the hepatocytes of an immunocompromised patient with hepatitis, suggesting

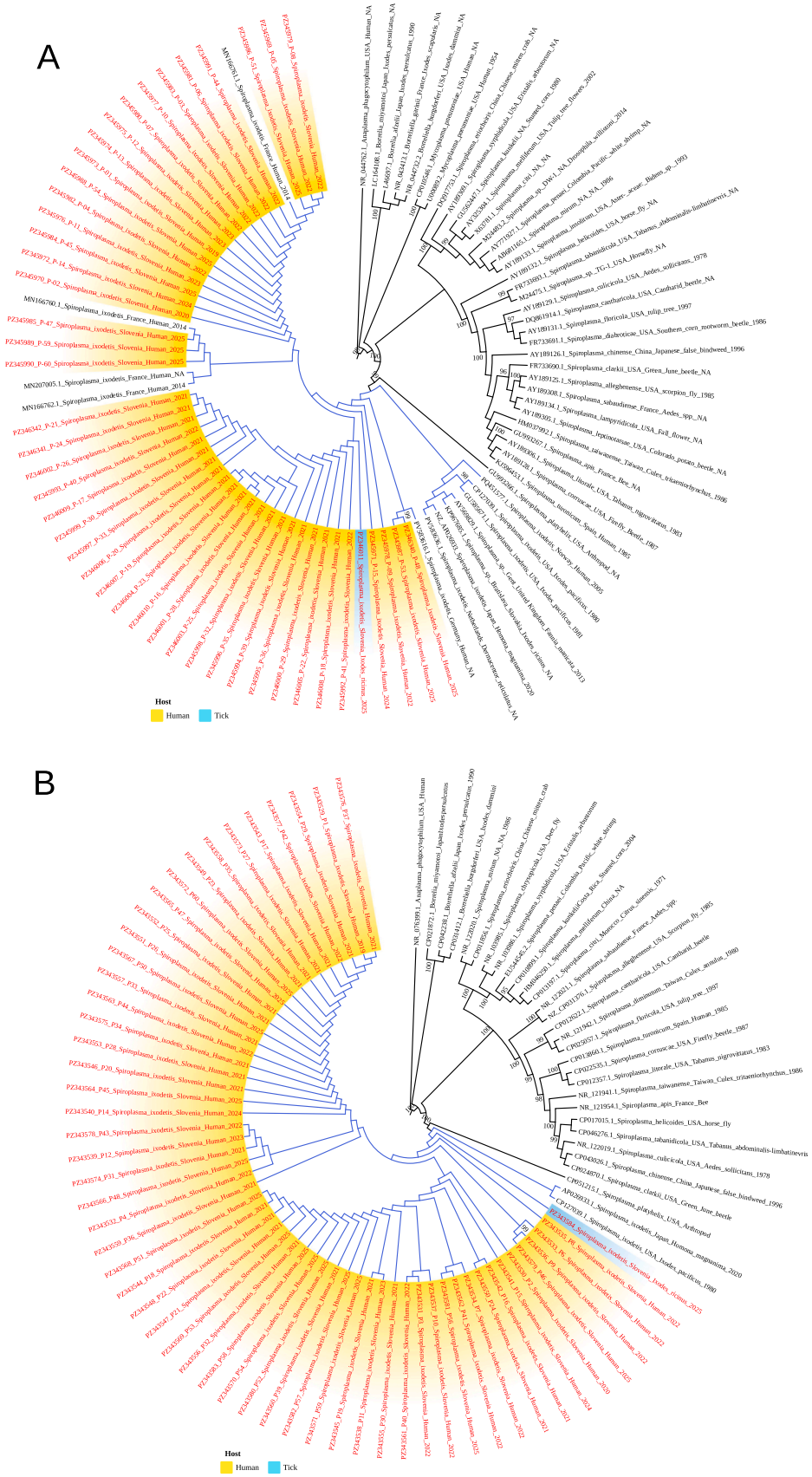

Fig. 3. Phylogenetic analysis of *S. ixodetis* sequences. A, Maximum likelihood tree of 45 full-length 16S rRNA consensus sequences, as well as one *S. ixodetis*–positive tick pool (Ti197/25) collected in Sodražica, Slovenia, in June 2025. B, Maximum likelihood tree of the 734 log fragment of the 23S rRNA gene obtained from 55 *S. ixodetis*–positive human samples and the positive tick pool (Ti197/25). For both targets, the maximum likelihood tree was constructed using our sequences, as well as reference genome sequences (*S. ixodetis* Y32, sHm), sequences of other *Spiroplasma* species, and *A. phagocytophilum* and *B. burgdorferi sensu lato* as outliers. Ultrafast bootstrap support values greater than 95 are indicated on the branches. Sample names labelled in red are sequences from this study; yellow blocks are sequences from human samples; cyan blocks are the sequence from the tick pool; and blue branches are *S. ixodetis* clusters.

localisation of *Spiroplasma* in the liver.[16] The latter seems to be further supported by a very recent publication from Farhat et al. who identified *S. ixodetis* in the liver of a kidney transplant patient who died due to fulminant hepatitis[22] and the findings in our patients, who typically had elevated liver enzyme activity (see Supplementary Table S4). Overall, clinical presentation in our patients corroborated the findings in both *S. ixodetis* systemic cases described so far, in which fever, elevated C-reactive protein (CRP) levels, thrombocytopenia, lymphopenia, and/or increased liver enzyme activity were reported.[16,18]

Limited data are available on the source of infection with *Spiroplasma* spp. which has been proposed to be either tick-bite related,[18] insect-sting related,[10] or of yet unknown origin[9,15–17,19] with implicit indication of vector-related infection.[17] In our study, 70% (42/60) of *S. ixodetis*–positive patients explicitly reported a tick bite, and all of them presented with the disease in months during tick activity in Slovenia. The 16S rRNA phylogenetic tree displayed limited intraspecific variability, reflecting the conserved nature of this marker and close genetic relatedness of detected cases. Although the 23S rRNA phylogenetic tree exhibited slightly greater internal branching, suggesting minor genetic diversification among isolates, the cases still clustered together. Moreover, phylogeny consistently shows a topology that clusters *S. ixodetis* independently of the host, thus demonstrating genetic coherence, which indicates a shared ecological and transmission link between ticks and humans. This information, coupled with the presence of genetically closely related *S. ixodetis* in a pool of *I. ricinus* ticks collected from a well-known tick-borne disease hotspot area in Slovenia, strongly suggests that ticks are the source of infection. However, a recent study suggested divergence between patient-derived *S. ixodetis* and tick-derived strains, but the comparison was based on only a limited number of sequences derived from naturally infected ticks and may not reflect the full diversity present in nature.[23] Consequently, Lager et al. reported a presumably low risk of *S. ixodetis* transmission from ticks to humans.[24] However, in our study, febrile patients presented to the hospital one to three weeks after the tick bite, whereas in the aforementioned study, the initial samples were collected only days after the tick bite, with follow-up samples obtained 3 months later. Differences in the diagnostic approaches used to detect *S. ixodetis*, together with the timing of sample collection, may explain the contradictory findings. Nevertheless, other potential sources of infection, such as mosquitos and other biting insects, need to be investigated in future studies.

In conclusion, this is the first study that demonstrates *S. ixodetis* as a frequent, but unrecognised cause of unlocalised fever in adult patients. Although further studies are required to definitively establish causation, these results point into the direction of *S. ixodetis* being the third most common tick-borne pathogen in Europe and call for a fundamental change in the guidelines for testing patients with an acute febrile illness without obvious localisation. The developed real-time PCR specifically tailored for human application shows superior performance to previous detection methods and can easily be used to test patients with the relevant clinical presentation.

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CRediT authorship contribution statement

Conceptualisation: MK, PB, NK, FS, TAŽ; Data curation: PB, RK, JS, SZ, AS, KRR; Formal analysis: MK, PB, RK, JS, SZ, AS; Funding acquisition: MK, TAŽ, MP, FS, PB; Investigation: MK, PB, RK, JS, KRR, SZ, AS, PP. Methodology: MK, RK, JS, KRR, SZ, AS, PP; Project administration: MK, PB, FS, TAŽ, MP. Resources: MK, PB, FS, TAŽ, MP; Software: SZ, AS, JS; Supervision: MK, PB, FS, TAŽ, MP; Validation: MK, PB, RK, NK, KRR; Visualization: RK, AS, SZ; Writing – original draft: MK, PB, RK, SZ, AS, FS; Writing – review & editing: all authors. All authors critically revised the manuscript and approved the final version of the manuscript.

Data availability

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at the European Nucleotide Archive (ENA) under the project label: PRJEB104317. Raw metagenomic data for individual samples can be found under the following ENA accession nos. ERS27947330-ERS27947344; 16S rRNA raw data under ENA accession nos. ERS27947346–ERS27947351, ERS27947353– ERS27947357, ERS27947359–ERS27947360, ERS27947362–ERS27947399, ERS27 947401–ERS27947407, ERS27947409–ERS27947410 and 23S rRNA raw data under ENA accession nos. ERS27947411–ERS27947413, ERS27947415–ERS27947417, ERS27947419–ERS27947467. The consensus 16S and 23S rRNA sequences can be found in the databases at the National Center for Biotechnical Information (NCBI) under GenBank accession nos. PZ345969–PZ346010, PZ346340–PZ346342 and PZ343529–PZ343583, respectively. The tick pool-derived 16S and 23S rRNA consensus sequences are available under GenBank accession nos. PZ346011 and PZ343584, respectively. The complete list of accession numbers is available in Supplementary Table S5.

Declaration of Competing Interest

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work no generative AI or AI-assisted tool has been used.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jinf.2026.106776.

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