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First case of brucellosis caused by an amphibian-type *Brucella*

Running title : 1st infection by frog Brucella in human

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ABSTRACT: We report the first case of brucellosis caused by an isolate whose genome is identical that of a frog isolate from Texas, demonstrating the zoonotic potential of amphibian-type *Brucella*. Importantly, with such atypical *Brucella*, correct diagnosis cannot be performed using routine serological tests or identification methods.

Brucellosis, an infectious disease caused by members of the *Brucella* genus, remains one of the most common bacterial zoonotic diseases worldwide and is endemic in most developing countries, mainly in areas with extensive farming [1]. *Brucella* are Gram-negative facultative intracellular bacteria that can infect many organs and soft tissues. *Brucella melitensis* (goats) is the predominant species causing human brucellosis, but *B. abortus* (cattle) or *B. suis* (swine) may also be involved. Transmission is commonly due to the consumption of unpasteurized dairy products or direct exposure to diseased animals. Metropolitan France is officially free from bovine brucellosis since 2005 and autochthonous human infections are extremely rare. Human brucellosis is a chronic debilitating infection with non-specific clinical presentation, often presenting as a flu-like syndrome. Symptoms occur after an incubation period of 2 weeks, or up to several months in some cases.

Mid-January 2019 (day 0), a French 28-years-old patient was hospitalized in the general hospital of Lorient (France) for an exploration of polyadenopathies associated with multiple pulmonary condensations.

Four months earlier, the patient had reported a left cervical lymphadenopathy, associated with general degradation in condition and weight loss (8 kg in 3 months). A cervical ultrasound performed on day -30 confirmed fluid accumulation in the left cervical region and revealed two others in the maxillary region. The patient also reported intense night sweats (without known fever) and a loose cough that resolved spontaneously. The patient, also affected by Wilson's disease, previously suffered from hypertension requiring multiple ligations of varicose veins. His treatment at day 0 was: propranolol, pyridoxine, D-penicillamine, paroxetine and hydroxyzine.

On admission (day 0), the patient had altered general status, moderate dyspnea without cough and night fever (39°C). Clinical examination confirmed a left cervical lymphadenopathy. Heart and lungs auscultations were normal. Initial laboratory tests (day 1) showed a C-reactive protein level of 37 mg/L, indicative of an active infection, but total leukocyte count of within the normal range ($5 \times 10^9/L$ with 60% polymorphonuclear cells.

HIV serology was negative. Blood cultures were negative after five days incubation. A cervico-thoracic and abdominal CT scan revealed multiple mediastinal adenopathies, pulmonary parenchymal condensations, cystic emphysematous lesions in the right upper lobe and splenomegaly (Figure S1).

On day 6, a left cervical lymph node was surgically removed, which triggered fever in the patient. Histopathologic examination of the excised tissue revealed an aspecific epitheliogigantocellular granuloma with caseous necrosis, without acid-fast bacilli (later confirmed by negative mycobacterial culture). Aerobic cultures of the tissue were positive after 48h incubation on Chocolate + polyvitex, Drigalski and Columbia + 5% sheep blood agar plates (bioMérieux). Colonies were about 1 mm in diameter, circular, glistening, smooth, non-pigmented and non-haemolytic. Culture of endobronchial aspiration from the patient in a Mycobacteria Growth Indicator Tube (Becton Dickinson) amplified bacteria that, upon sub-culturing on a Columbia plate, gave colonies with similar appearance. Bacteria were Gram-negative coccobacilli and showed positive reactions for catalase and cytochrome-oxidase, but negative result for indole production. The bacterial isolate was analyzed by MALDI-TOF MS using a Biotyper (Bruker Daltonics) with the version 7 (V7) of the IVD (*In Vitro* Diagnostics) database. The identification result was *Ochrobactrum anthropi* (score 1.49). Because of the low score and inconsistency with phenotypic observations, the isolate was re-analyzed using Bruker's Security-Relevant Library database and the identification result was *Brucella melitensis* (score 2.23). A treatment with oral doxycycline (200 mg once daily) and rifampicin (15 mg/Kg/day) was thus initiated and the isolate sent to the national reference centre (CNR *Brucella*) for confirmation of identification and further analysis. MALDI-TOF MS re-analysis using a Vitek MS (bioMérieux) with the IVD database V3.2 confirmed that the isolate belongs to the *Brucella* genus. However, the use of a recently developed, more complete, RUO (Research Use Only) database [2] rather suggested that it was a *B. inopinata* (score 99.9%). A blood sample drawn on day 1 was also submitted to several *Brucella* serological tests: Rose Bengal (Brucelloslide-Test, bioMérieux), Wright agglutination

(*Brucella* Wright, Bio-Rad), ELISA (Virclia IgM or IgM, Vircell) and immunocapture test (BrucellaCapt, Vircell). All tests were negative.

After 6 weeks of antibiotic therapy, the patient reported resolution of his malaise and fatigue but presented a left cervical abscess under a surgical scar. Culture of an aspirate from this abscess was negative, but *Brucella* DNA was detected by PCR amplification of the *Brucella*-specific sequence IS711 [3]. The persistence of deep-seated lymphadenopathy associated with multiple pulmonary condensations was confirmed by a CT scan showing an unsatisfactory progression. The antibiotic treatment was thus prolonged for a total of 3 months, to which the patient responded favorably, remaining clear of symptoms 6 months after the end of treatment.

An epidemiological survey was conducted to try identifying the origin of the infection. The patient declared no travel abroad in the past five years and no consumption of unpasteurized dairy products. However, as part of his professional activity (animal keeper specializing in exotic animals), he reported many direct contacts with reptiles, amphibians, rodents or birds, several imported from abroad.

The bacterial isolate was characterized using a Bruce-Ladder multiplex PCR [4] on genomic DNA and phylogenetic positioning based on MALDI-TOF MS spectra. This revealed that this isolate is most similar to the *B. inopinata*-like isolates BO2 and B13-0095, isolated from a patient with severe pulmonary infection [5] and from a frog [6], respectively (Figures S2 and S3). The isolate, thereafter named BO3 (for *Brucella inopinata*-like 3), showed susceptibility to all tested antibiotics, as for *B. melitensis* and the other *B. inopinata*-(like) strains (Table S1). Several features however clearly distinguished BO3 from classical *Brucella* spp. First, slide agglutination using a polyclonal serum anti-*Brucella* (ANSES, Maisons-Alfort, France) was negative. We also found that BO3 is highly motile, like other *B. inopinata*-(like) strains [6], and flagellated (Figure S4), which is completely novel for this bacterial genus.

Sequencing of BO3 genome revealed two chromosomes of 2,181,202 bp and 1,197,108

bp, respectively, with a GC content of 57.11%, similar to other *Brucella* species. No plasmid was detected. A genome-based phylogenetic tree confirmed that BO3 belongs to the group of ‘atypical’ *Brucella*, together with the human isolates BO1 (USA, 2005 [7]) and BO2 (Australia, 2007 [5]) and a growing number of frog isolates [8], and is extremely closely related to the frog isolate B13-0095 [6] (Figure 1). Annotation of the BO3 genome using PATRIC (<https://www.patricbrc.org>) identified the *virB* operon (encoding a type IV secretion system) on chromosome 2. As with strains BO2 and B13-0095, the *wbk* operon (encoding the perosamine-based O-antigen in classical *Brucella* species) is absent from BO3 genome and, instead, a gene cluster encoding a system for L-rhamnose uptake and catabolism is present, suggesting an atypical lipopolysaccharide. BO3 DNA also contains a cluster of genes involved in ectoine catabolism (Figure S5), a genetic feature specific to amphibian-type *Brucella* [6,8]. When calculating genome-to-genome distance, the DNA-DNA hybridization (DDH) estimate between strains BO3 and B13-0095 was 99.9%, indicating that these genomes are identical. As a comparison, the DDH estimate between BO3 and BO2 was 89.8%, 84.9% with BO1, 83.3% with 09RB8471 (African bullfrog [9]), 83.2% with 141012304 (bluespotted ribbontail ray [10]), 81.5% with 10RB9215 (African bullfrog) and 81.4% with 09RB8910 (African bullfrog). Altogether, our analyses indicate that this new strain BO3 is identical to the *B. inopinata*-like strain B13-0095 that was isolated from a Pac-Man frog in Texas in 2010 [6].

This is thus the first report of human brucellosis caused by an amphibian-type *Brucella*. The scarcity of reports may be due to the difficulty to identify them as *Brucella* spp. Because of their rapid growth rate on plates (< 24h) and because phenotypic identification tests (such as API 20NE) misidentify them as *Ochrobactrum* spp. with a high confidence level [6,11]. Moreover, since some strains have an LPS that is chemically and antigenically different from that of classical *Brucella*, infection cannot be detected using commonly available serological tests and the isolates cannot be identified using specific typing serum. Finally, when using MALDI-TOF MS as an identification method, it should be noted that the Biotyper (Bruker),

with its Security Relevant database, and the Vitek MS (bioMérieux), with its most recent IVD database (\geq V3.2), would identify such atypical isolates as *B. melitensis* or *Brucella* spp., respectively. Correct identification as *B. inopinata*(-like) is currently only possible using the latest version (\geq V4.15) of the RUO database Saramis on a Vitek MS [2].

The animal reservoir(s) of *B. inopinata*(-like) strains remain to be identified. Isolation from different animals (either wild caught or kept in zoological collections, pet store or by private breeder [8]) on several continents suggests that these bacteria are widespread. Recent isolation from a ray also suggests that all vertebrates (including fish and reptiles) may be suitable hosts. We could not detect traces of *Brucella* in cultures of samples taken from the patient's pets (mainly snakes), suggesting that exposure occurred during his past professional activity as an exotic animal keeper (notably, the patient declared being in contact with Pac-Man frogs). We speculate that *B. inopinata*(-like) bacteria may be part of the normal flora of some animals and become opportunistic pathogens for their host upon unfavorable conditions such as stress caused by transport (importation of wild-caught animals), changes in breeding facilities (*i.e.* quarantine protocols) or contact with other species creating opportunities for cross-species infection. Also, pathogenicity for humans may be linked to lowered immunocompetency.

Medical practitioners and diagnostic laboratories should be aware of the existence of these pathogens. Prevention measures should be taken, especially for exotic animal keepers and veterinarians, who should limit direct contact with potentially infected animals.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: All authors do not declare any conflict of interest.

Ethical approval: Publication of case reports does not require approval by an Institutional Review Board.

Informed consent: The patient gave his written informed consent for the publication of this case report.

Nucleotide sequence accession numbers and deposition

The genome sequences of isolate BO3 are available from the GenBank database under accession numbers CP047232-CP047233 (BioProject PRJNA597040).

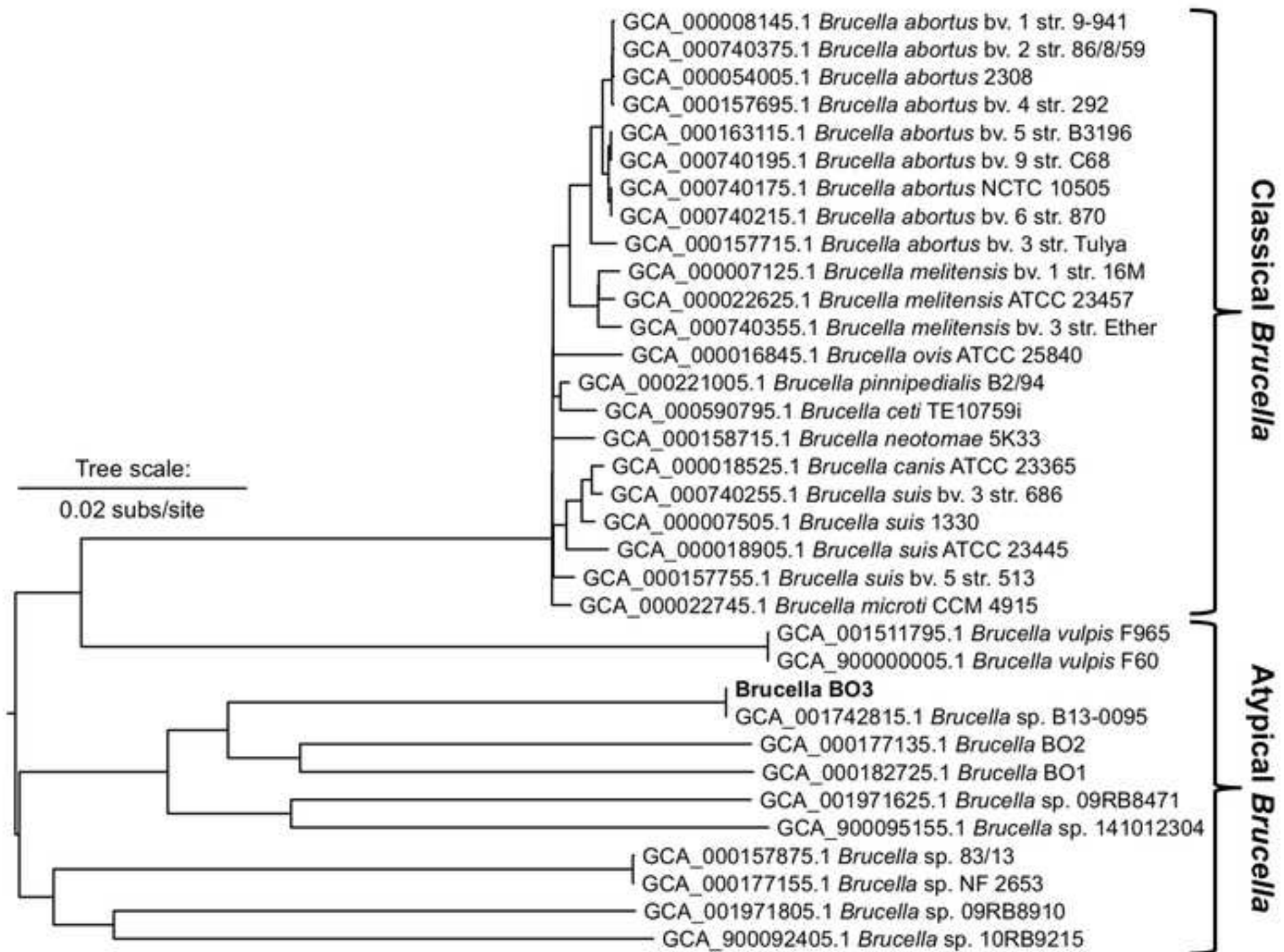
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FIGURE LEGEND

Figure 1: The amphibian-type isolate BO3 belongs to the emerging group of atypical *Brucella*. BO3 genomic DNA was extracted (DNeasy UltraClean Microbial Kit, Qiagen) and sequenced using MinION (Oxford Nanopore Technologies) and Illumina MiSeq (Illumina) platforms. Sequence reads were *de novo* assembled with Unicycler v0.4.7, processed with Circlator v1.5.5 and polished with Pilon v1.23) resulting in a high quality genome. A genome-based tree was then constructed using the reference strains of all *Brucella* species and selected ‘atypical’ strains. Assemblies were aligned to a reference genome with NUCmer; core genome single nucleotide polymorphisms were called within NASP; and a maximum likelihood phylogeny was inferred with IQ-TREE. This phylogenetic tree shows clustering of BO3 within the group of “atypical” *Brucella*, together with *B. inopinata* BO1 [7], *B. inopinata*(-like) BO2 [5], and isolates from Australian rodents (NF2653 and 83/13 [11]), red foxes (F60 and F965 [12]), a bluespotted ribbontail ray [10] and frogs (B13-0095, 09RB8910, 10RB9215 and 09RB8471 [6,9]).



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SUPPLEMENTARY MATERIAL

Figure S1: Initial CT scan showing hilar and subcarinal adenopathies (A) and parenchymal condensations (B).

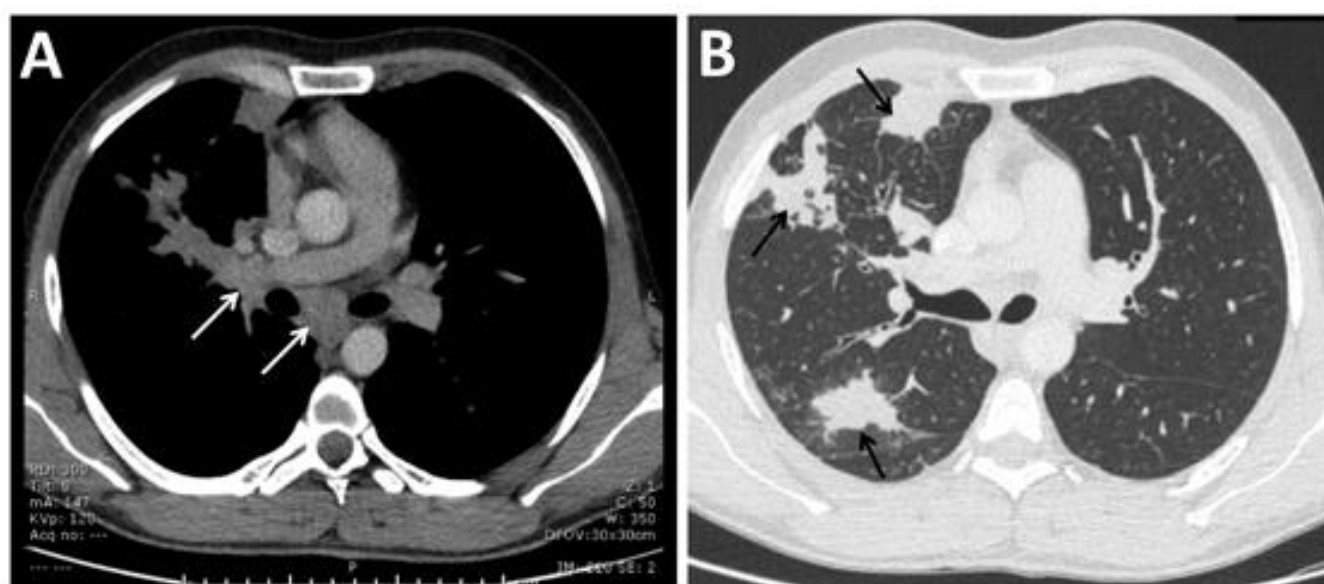


Figure S2: Identification of the patient bacterial isolates as *Brucella. inopinata*-like. Bruce-Ladder multiplex PCR was performed on two bacterial isolates BRSO-2019-086 and BRSO-2019-089, recovered after puncture of two different lymph nodes in the patient at day 6 and -7, respectively. Bruce-Ladder profiles were compared to classical (*B. melitensis*, *B. abortus*, *B. suis* and *B. canis*) or atypical (*B. inopinata* strain BO1 or *B. inopinata*-like strains BO2 and frog B13-0095) *Brucella* isolates after agarose gel electrophoresis. Left = molecular-weight size marker (size in bp). Ø = negative control.

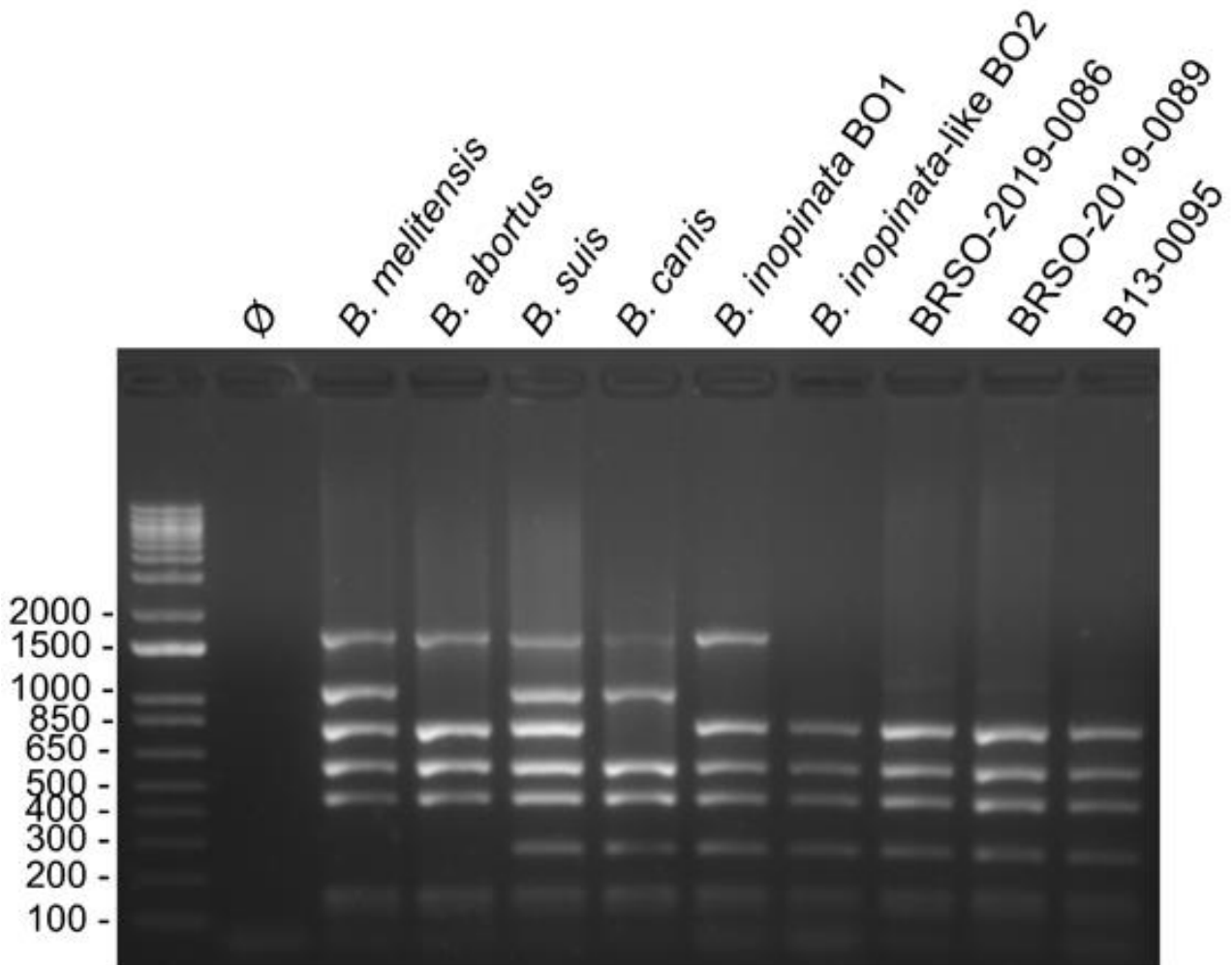


Figure S3: Proximity of MALDI-TOF MS spectra. Dendrogram constructed from MALDI-TOF MS spectra using SARAMIS (bioMérieux). Similarity between spectra is quantified by the proportion of common masses; the dotted line (65%) indicating a threshold above which bacteria are considered as significantly different. This representation shows the distance between *Brucella* strains belonging to the classical or to the atypical clade of this genus. This highlights clustering of the two patient isolates BRSO-2019-086 and BRSO-2019-089 (arrows) with the group of “atypical” *Brucella*, and confirms the clonality of these two isolates, which were thereafter considered as a single strain named BO3 (for *Brucella inopinata*-like 3).

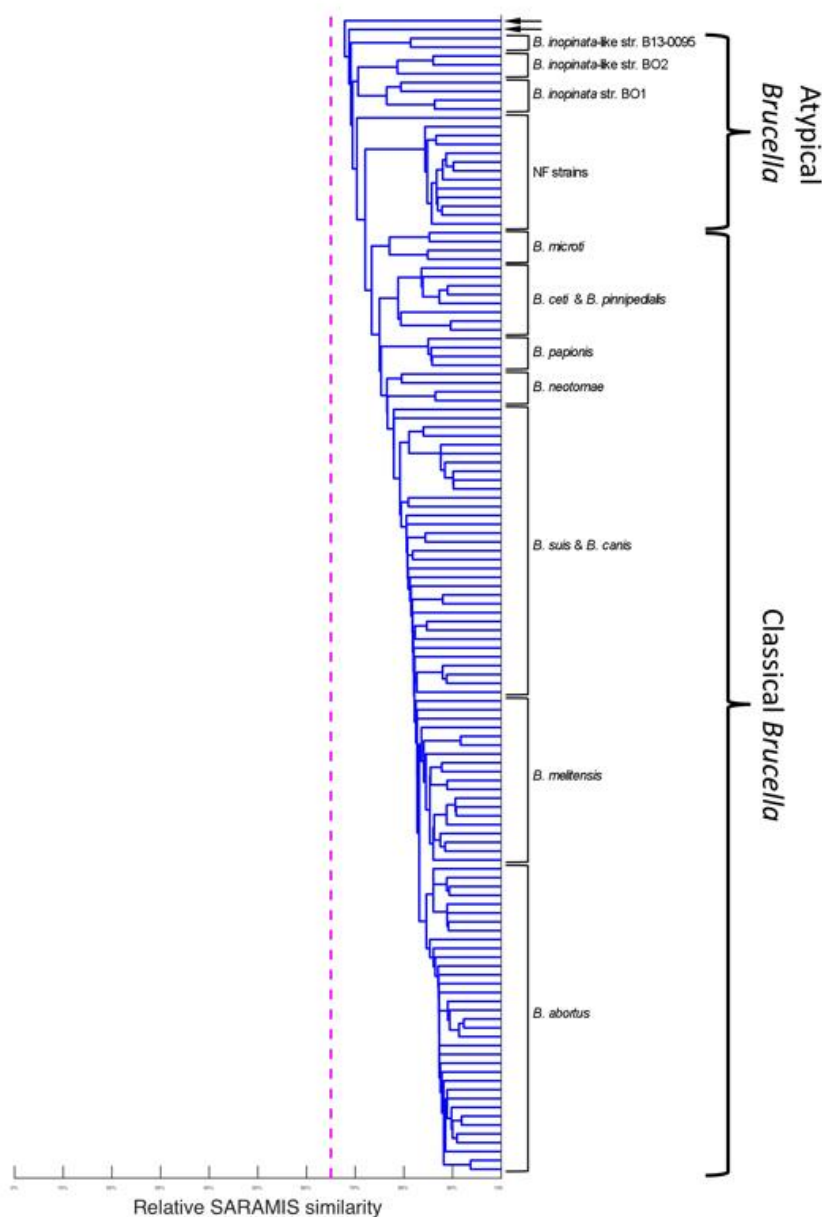


Table S1: Antimicrobial susceptibility tests by disk diffusion assay. Bacterial isolates were plated on Mueller-Hinton agar with 5% sheep blood (bioMérieux), as established by the Clinical Laboratory Standards Institute and considering suggestions for Gram-negative bacteria. The cut-offs (in brackets) and the measured inhibition diameters are indicated in mm. 16M = *B. melitensis* biovar 1 reference strain, BO1 = *B. inopinata* clinical isolate [1], BO2 = *B. inopinata*-like clinical isolate [2], BO3 = *B. inopinata*-like clinical isolate (this case), B13-0095 = *B. inopinata*-like frog isolate [3].

	Rifampicin (≥ 18)	Tetracycline (≥ 15)	Gentamycin (≥ 17)	Amikacin (≥ 16)	Trimethoprim + Sulfamethoxazol (≥ 14)	Ciprofloxacin (≥ 24)	Ofloxacin (≥ 22)	Amoxicillin (≥ 19)	Amoxicillin + Clavulanic acid (≥ 19)
16M	40	45	32	32	42	40	32	52	49
BO1	32	43	23	22	28	35	32	40	40
BO2	32	50	24	24	20/25	24/24	24/23	50	42
BO3	30	40	22	22	16/25	30/30	30/26	40	32
B13-0095	32	40	22	22	15/18	30/29	24/23	40	36

Figure S4: *Brucella* B03 isolate is flagellated and motile. A) Swimming assays on soft-agar plates showing that B03 is motile. As a control, strains B01 and B02 are also motile while *B. melitensis* strain 16M (classical *Brucella*) is not, in agreement with previous observations [3]. B) Visualization of B03 by electron microscopy revealing bacterial cells with a mean average diameter of 0.5 μm and a length of 1 μm , some of them with a polar flagellum (scale bar = 500 nm).

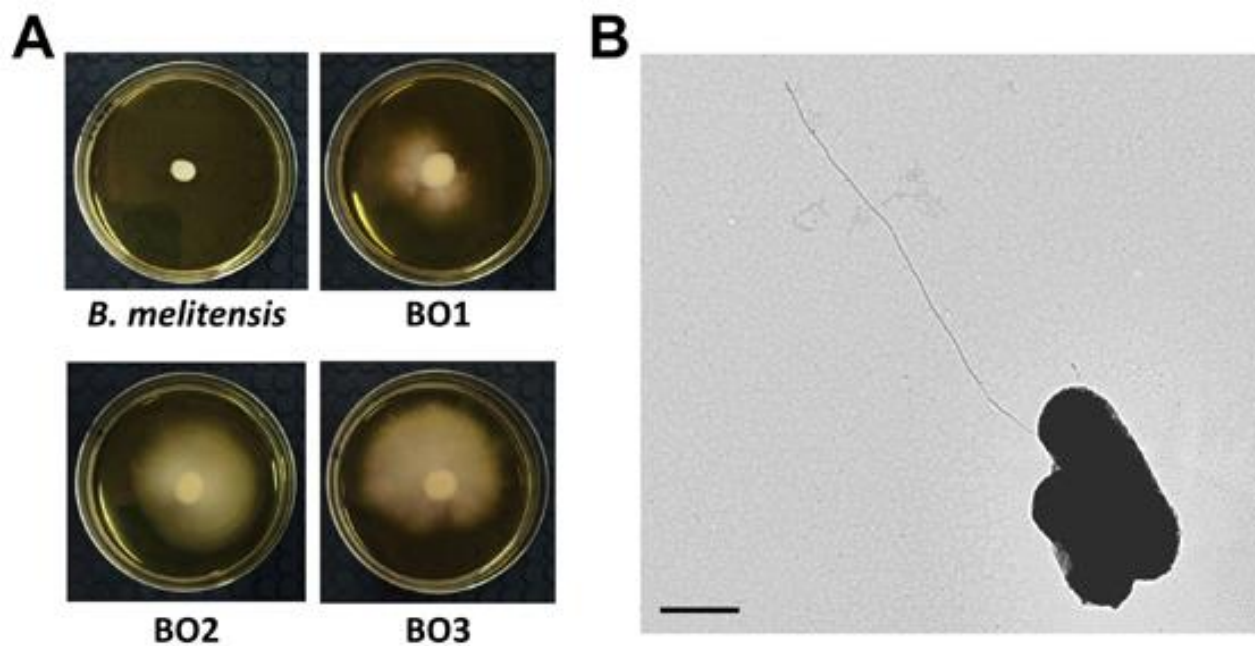
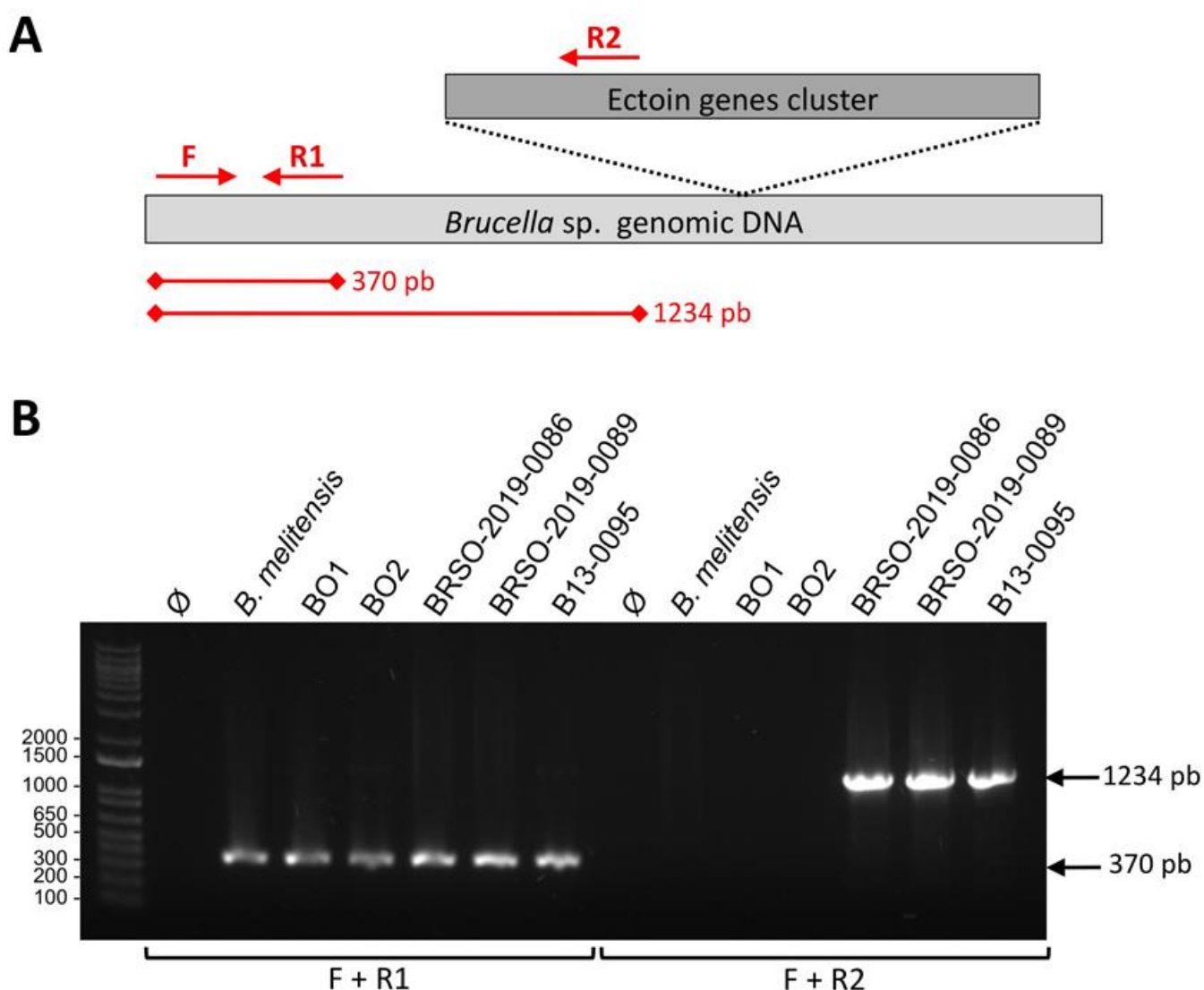


Figure S5: Detection of the Ectoin genes cluster by PCR. A) Specific PCR strategy: two independent reactions were performed using the primers pairs F (5'-actggctcttctcaagac-3') and R1 (5'-accgacaacatcgcaatc-3') or F and R2 (5'-gcaagttcttcgacccatc-3') to amplify either a 370 bp fragment common to all *Brucella* species (as a positive control) or a 1234 bp fragment when the bacterial genomic DNA contains the Ectoin genes cluster, respectively. B) PCR results for the two patient's isolates (BRSO-2019-086 and BRSO-2019-089) compared to other *Brucella* isolates (*B. melitensis* 16M, *B. inopinata* strain BO1, *B. inopinata*-like strains BO2 and frog B13-0095) after agarose gel electrophoresis. Left = molecular-weight size marker (size in bp). Ø = negative controls.



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