Investigation of *Brucella* spp. presence of amphibians collected from some regions in Türkiye

ABSTRACT

Brucellosis is a zoonotic disease that causes economic losses in many countries worldwide, especially in livestock. Firstly, the African bullfrog and then *B. inopinata* and *B. microti*-like strains have been identified in various frog species worldwide. This study aimed to determine the presence of *Brucella* in amphibian frogs for the first time in Türkiye by bacteriological and molecular methods. Internal organ samples (spleen, liver, lung, kidney, etc.) of 150 frogs from different regions of Türkiye (Edirne/Ipsala (53 pieces), Adıyaman/Kâhta (97 pieces)) were used for the isolation of *Brucella* spp. As a result of *Brucella* genus-specific PCR (Polymerase Chain Reaction) and Multiplex PCR methods performed with these extracts, no positivity was detected in the frog samples taken from Edirne province, while in 4 of the frog samples taken from Adıyaman province, bands of approximately 250, 600, 700, 1000, 1500 and 3000 bp were observed in Multiplex PCR. *Sphingomonas paucimobilis* (*S. paucimobilis*) was identified by analysing the culture obtained from these samples with VITEK®2:Healtcare. As a result, the presence of *Brucella* spp. could not be detected both bacteriologically and molecularly in the study samples. However, observing similar multiple bands in multiplex PCR suggested that this bacterium and *Brucella* species are phylogenetically close. It was determined that *S. paucimobilis*, which belongs to the same class as *Brucella* species under the Alphaproteobacteria, may be dangerous for people who work on farms where frogs are raised for human consumption. This is because the bacteria can cause opportunistic infections, particularly in immunocompromised humans. Therefore, it may be imperative to take the appropriate precautions.

Keywords: Bruce-ladder, *Brucella* spp., Frog, Isolation, PCR, *S. paucimobilis*

INTRODUCTION

Brucellosis, also known as Malta fever, is a zoonotic infection caused by *Brucella* species, which are facultative, intracellular, immobile, aerobic, and Gram-negative coccobacilli (Buttigieg et al., 2018). Brucellosis is a disease characterized by reproductive disorders with a wide range of hosts and causes significant economic losses, especially in livestock. It is recognized as one of the most important bacterial zoonoses worldwide, with 500.000 human cases per year (Godfroid et al., 2005). Since there is currently no approved human vaccine, treatment is difficult and prolonged and carries the risk of recurrence, it is essential to update and increase the knowledge about the pathogenesis, diagnosis and treatment of brucellosis to control and manage this infection, especially in endemic areas (Amjadi et al., 2019). In addition to the six classical species affecting terrestrial mammals, the diversity of animal hosts has been expanded to include marine mammals and primates, bringing the number to 12 (Jaÿ et al., 2020; Whatmore et al., 2014). This classification is defined according to host preference, reproduction and biochemical properties (Scholz et al., 2008; WOAH, 2018).

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Research Article

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In recent years, more atypical *Brucella* spp. have been isolated from cold-blooded animals. Frogs are the cold-blooded animals from which the most isolations are made (Eisenberg et al., 2012; Leclercq et al., 2020; Mühldorfer et al., 2017). *S. paucimobilis* is a Gram-negative bacillus with a single polar flagellum, aerobic, yellow-pigmented, non-fermenting glucose, oligotrophic, non-spore-forming, non-spore-forming Gram-negative bacillus, which is very closely related to the genus *Brucella* and is reported to be widely distributed in natural and mainly aquatic environments. *Sphingomonas* is considered to be an opportunistic pathogen, and although it is rarely reported in clinical settings, it has been reported to be closely associated with meningitis cases (Tai and Velayuthan, 2014; Walayat et al., 2018). It has been reported to grow in distilled water, haemodialysis fluids and sterile drug solutions. Since it has been reported that it can be transmitted, especially to people with chronic diseases or immunocompromised diseases, it is essential for public health (Göker et al., 2017). *S. paucimobilis* was isolated from frogs for the first time in Türkiye (Dökenel and Özer 2019).

The natural host range of Brucellosis disease has expanded to amphibians in recent years due to reports of some atypical *Brucella* spp isolated from frogs. In France in 2017, a *Brucella* strain was isolated for the first time from animals in a frog (*Pelophylax ridibundus*) farm produced for human consumption and identified as *B. microti*-like. Following this first isolation, *B. microti*-like strains were isolated in the samples taken from the frog farms and their environment, indicating that the agent can survive in the host environment. This situation reveals the zoonotic and pathogenic potential of atypical *Brucella* species and shows that they may pose a possible risk to consumers and workers (Jaý et al., 2018). This strain from the frog showed genetic and phenotypic characteristics similar to the isolates obtained from the field mouse (*Microtus arvalis*) in the Czech Republic (Hubálek et al., 2007). In addition, atypical *Brucella* isolations from various frog species such as African bullfrog (*Pyxicephalus edulis*) (Eisenberg et al., 2012), big-eyed tree frog (*Leptopelis vermiculatus*) (Fischer et al., 2012), white tree frog (*Litoria caerulea*) (Whatmore et al., 2015) were realised (Jaý et al., 2018). Significant research is being conducted to expand the current knowledge on atypical *Brucella* isolated from amphibians worldwide and to address the challenges faced (Mühldorfer et al., 2017). It has been reported that Türkiye exported an average of 642 tons of edible frogs. The number of farms in Türkiye has increased over the years due to commercial frog breeding and its great potential as a food source. The demand for frogs is rising and global frog consumption is rising as a result of the decline in frog populations. Most nations that import wild and farmed frogs are Türkiye as well as Indonesia, China, Thailand, India, and Vietnam (Şimşek et al., 2022).

In this study, organ materials (liver, spleen, spleen, lung, heart, kidney, ovaries and skin) of 150 frog specimens of different species and sizes collected from Edirne/İpsala and Adıyaman/Kâhta provinces/districts were investigated for *Brucella* spp, and it was aimed to obtain data on the presence of *Brucella* in amphibia for the first time in Türkiye.

MATERIALS AND METHODS

Animal materials

In this study, organ materials (liver, spleen, lung, heart, kidney, ovaries, and skin) of a total of 150 frog specimens (53 examples from farms in the Edirne/İpsala region and 97 samples from nature from Adıyaman/Kâhta region) of different species and sizes were used as examination samples for the diagnosis of *Brucella* spp. in the spring months of 2022-2023.

Culture and biotyping

Tryptic Soy Agar (TSA) (CM0131, Oxoid) was prepared according to the manufacturer's protocol and sterilized by autoclave at 121 °C for

15 min. After the medium was cooled to 50-55 °C, Farrell's medium was prepared by adding *Brucella* Selective Supplement (SR0083A, Oxoid) and inactivated bovine serum (5%) (Biochrom, S0115, Germany) under sterile conditions to the prepared TSA medium. Tissue homogenates prepared from organ materials of frog samples were simultaneously inoculated into Farrell's agar and enrichment liquid medium containing vancomycin (20 µg/ml), amphotericin B (1 µg/ml), 1% dextrose and 5-10% serum. The cultured sample materials were incubated in an oven containing 5-10% CO2 under microaerophilic conditions for six weeks. During this period, passages were made on TSA and Farrell's medium. Classical bacterial methods were applied for the diagnosis of *Brucella* spp. (Alton et al., 1988; WOAH, 2022). DNA extraction was performed from the isolates studied for *Brucella* spp.

DNA extraction

A commercial isolation kit (High Pure FFPET DNA Isolation Kit, 06650767001, Roche) was used for DNA extraction from suspected cultures and tissue homogenates simultaneously.

Reference materials

B. melitensis Rev 1 strain, Tbilisi, Izatnagar, and R/C phages and monospecific sera (A, M) were obtained from Harran University, Faculty of Veterinary Medicine, Department of Microbiology Laboratory.

Species specific and multiplex PCR (Bruce-Ladder)

For the genus-specific DNA amplification, a 223 bp sequence present on a gene encoding the BCSP31 protein, which is present in all *Brucella* species and weighs 31 kDa, was considered by the previously described protocol (Baily et al., 1992). To amplify this target sequence, primers B4 F (5′ TGG CTC GGT TGC CAA TAT CAA 3′) and B5 R (5′ CGC GCT TGC CTT TCA GGT CTG 3′) (Sigma Aldrich) were used. The multiplex PCR method was performed according to the method reported by Mayer-Scholl et al., 2010. The obtained DNA extracts were prepared with 2x Taq PCR Mastermix (206143, Qiagen) protocol, with a total volume of 25 µl in each tube, containing 12.5 µl Qiagen master mix, 0.2µM of each of 9 primer pairs, nine µl water and 1 µl template DNA. The contents of the mixture were amplified in a Thermal Cycler (Thermo Fisher). The cycles determined for amplification were performed by the commercial mastermix content. For this purpose, after initial denaturation at 95 ºC for 15 min, amplification was completed at 94 ºC for 30 s, initial binding at 58 ºC for 90 s, 72 ºC for 3 min (first synthesis), and final synthesis at 72 ºC for 10 min, for a total of 30 cycles. At the end of the process, amplicons were subjected to electrophoresis (Thermo Scientific EC300 XL). Agarose gel (Sigma, A9539) was prepared at a concentration of 1.5%. At the end of the procedures, specific bands in the gel were investigated with a gel imaging device (Major Science UVCI-1100).

RESULTS

Bacteriological analyses were performed according to the standards set by WOAH (WOAH 2022). All cultures were incubated in Farrell's broth at 37 °C, 5-10% CO2 for six weeks. During this period, Farrell's agar was inoculated weekly, and as a result of the inoculations, specific honey-colored, smooth-looking suspicious colonies similar to *Brucella* spp. were found. The suspicious colonies were subjected to an agglutination test with A and M monospecific sera, but no agglutination was observed. These isolates were also not lysed by Tblisi, Izatnagar and R/C phage. As a result of the *Brucella* spp. and Bruce-Ladder PCR method performed on suspected colonies isolated from the cultured and incubated media and amplicons obtained by DNA extraction from tissue homogenates simultaneously, no positivity was

received in the samples collected from the Edirne/Ipsala region. As a result of the Bruce-Ladder PCR method performed on samples collected from the Adıyaman/Kâhta region, approximately 250, 600, 700, 1000, 1500 and 3000 bp bands were detected in 4 of 97 samples, but no positivity was obtained in any of the samples as a result of the genus-specific PCR for *Brucella* spp. (Figure 1). As a result of the identification (VITEK®2:Healtcare automatic identification device) of the isolates exhibiting different band profiles as a result of multiplex PCR analysis, samples 61, 62, 63 and 129 were identified as *S. paucimobilis*.

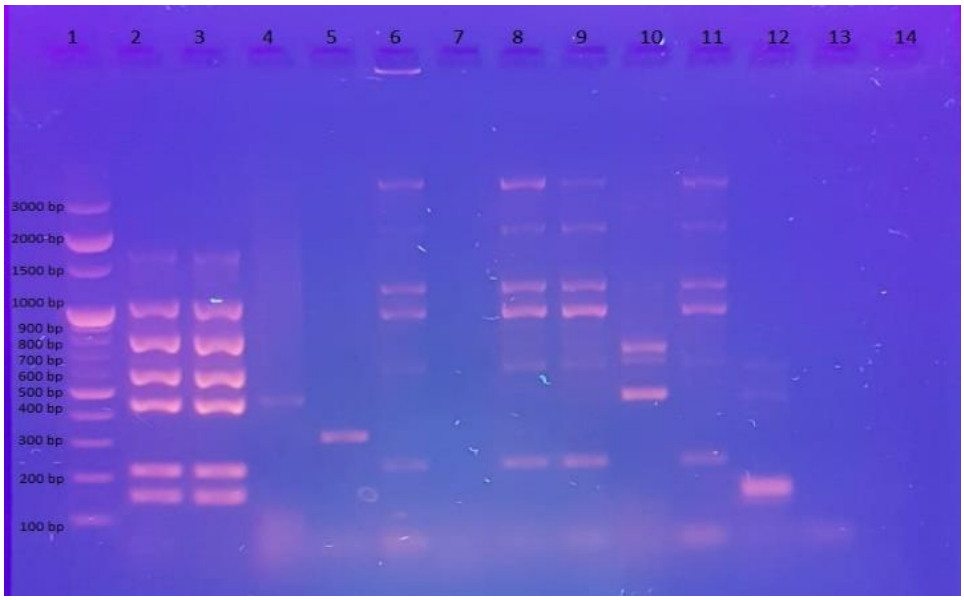

Figure 1. Leadder (Qiagen GelPilot Mid Range katalog no: 239135) 2,3: Positive control (B. melitensis Rev 1), 6,8,9,11: Sphingomonas paucimobilis, 4,5,7,10,12,13; Negative samples, 14;Negative control

DISCUSSION

Until recently, the genus *Brucella* was thought to be a clonal group of genetically identical bacteria isolated only from mammals, but the recent isolation of *Brucella* group bacteria from cold-blooded animals such as frogs, lizards and fish has radically changed this idea. In contrast to the known classical *Brucella* species, the recently reported species isolated from exotic frogs have a different ecology, are genetically diverse and may represent several new *Brucella* species (Scholz et al., 2016; Whatmore et al., 2007). This study is essential as the first study investigating *Brucella* spp. in frogs in Türkiye. *Sphingomonas* is very closely related to *Brucella*. It is reported that *S. paucimobilis* shares genes related to intracellular vitality with *Brucella* species, and genes related to adhesion and movement are co-expressed with *Legionella* species (El Beaino et al., 2018). Although considered to have low virulence, *S. paucimobilis* can cause infections in chronically ill and immunocompromised hosts (Göker et al., 2017).

Atypical *Brucella* isolates have been isolated from many frog species and identified as the cause of localized infection, including skin and soft tissue abscesses, panophthalmitis, spinal arthropathy as well as systemic disease (Mühldorfer et al., 2017). Systemic lesions were found in the skin and internal organs of some healthy frogs used in this study. Some researchers have isolated *Brucella* spp. from various frog species for cultural and molecular studies (Jaÿ et al., 2020; Latheef et al., 2020). The present study determined that the suspicious colonies obtained from various tissue samples due to the culture method were not identified as *Brucella* spp. no positivity was obtained in the PCR study performed at the genus level (Bcsp31). However, the detection of multiple bands in the species level (Bruce-Ladder) study indicates that *Brucella* spp. can be colonized in amphibians by other organisms in the same

genus and can continue its presence in their environment. The cultures analyzed on the VITEK®2:Healtcare automatic identification device were identified as *S. paucimobilis*, included with *Brucella* spp. in the *Alphaproteobacteria* class. It has been reported that *S. paucimobilis* has been isolated from many patients as case reports in Türkiye (Aşkın et al., 2022; Göker et al., 2017; Gün et al., 2014; Özekinci et al., 2022). Although many studies are isolated from humans in hospitals, *S. paucimobilis* was identified for the first time in frogs in Türkiye. In a study conducted in Germany to investigate the presence of atypical *Brucella* in amphibians, *S. paucimobilis* was found in 11 of 27 isolates and *Ochrobactrum* in 16 of 27 isolates (Mühldorfer et al., 2017). This result, which is compatible with our study, suggests that *S. paucimobilis* is in close association with *Brucella* species.

Occupational exposure through direct contact with infected animals and foodborne transmission through consumption of raw animal products are recognised as the main routes of brucellosis transmission to humans (Corbel et al., 2006). For this reason, *Brucella* spp. is important in terms of a possible risk for workers and consumers due to the pathogenic and zoonotic potential of similar atypical species. There are frog breeding farms in our country, which are seen as luxury consumption food and are exported abroad in large quantities or for domestic consumption (Gülçiçek, 2021). It is essential to determine the prevalence of *B. microti* and *B. inopinata*-like organisms among the frog population raised here and to investigate their presence in the farm environment (Jaý et al., 2018).

CONCLUSION

In this study, *Brucella* spp. could not be isolated from amphibians. However, both the relatively small sample size and the fact that the clinical samples were taken from healthy frogs may have caused this result. Future studies aim to investigate the presence of *Brucella* spp. in amphibians, especially by taking more samples with lesions from different locations.

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Ethical statement or informed consent: This research was approved by the Ethics Committee of Harran University Faculty of Veterinary Medicine (HRÜ-HADYEK, Ref No: 2020/007/01-2, Date: 25/12/2020).

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