



# Isolation and Identification of Two *Brucella* Species from a Volcanic Lake in Mexico

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Received: 30 January 2020 / Accepted: 25 August 2020 / Published online: 8 September 2020  
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## Abstract

Brucellosis is a zoonosis caused by bacteria of the *Brucella* genus. Any source of contamination that could be infectious must be monitored to reduce the risk of exposure to brucellosis, so the purpose of this work was to determine the presence of *Brucella* spp. on surface water and tilapia (*Oreochromis niloticus*) skin from a volcanic lake in Mexico. A seasonal sampling during 2016–2017 was carried out at fifteen specific sites for water sampling and five sites for the collection of tilapia fish. From all water and fish samples tested, we found only three isolates of *Brucella* species. We isolated and identified *B. abortus* from surface water through bacteriological and molecular techniques, and *B. abortus* and *B. suis* from the same tilapia skin sample. The isolated strains likely came from breeding animals that are common to the region, such as infected pigs or cattle with *Brucella abortus* or *B. suis*, respectively. A similar finding has not been reported in a water from volcanic lake or tilapia fish in Mexico. We concluded that *B. abortus* and *B. suis* are present on the surface water of the volcanic lake and tilapia skin as possible contaminants derived from biological material from cows and pigs carrying this bacterium.

**Electronic supplementary material** The online version of this article (doi:<https://doi.org/10.1007/s00284-020-02184-z>) contains supplementary material, which is available to authorized users.

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## Introduction

Brucellosis is an infectious disease caused by bacteria of the *Brucella* genus transmitted from animals to humans [1]. The disease is transmitted from animals to humans via ingestion (unpasteurized milk or dairy products), inhalation, or exposure through the conjunctiva or skin abrasions [1, 2]. Brucellosis predominates in Mediterranean countries, the Middle East, and Latin America [3]. Based on the host preference and pathogenicity, twelve species are recognized within the genus *Brucella*: *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis*, *B. neotomae*, *B. ceti*, *B. pinnipediae*, *B. microti*, *B. inopinata*, *B. papionis*, and *B. vulpis* [1, 4]. *Brucella melitensis* is one of the most virulent species, followed by *B. suis* and *B. abortus* [5, 6]. Brucellosis continues to be a reemerging zoonosis worldwide and in Mexico. Although the Mexican government has implemented a campaign for brucellosis control and eradication since 1995, there was an increase in incidence from 2000 to 2011 [7]. The incidence of bovine brucellosis increased from 1 to 15%, and in the case of goats, it increased from 2 to 7% [7]. Mendez et al. reported that in this same period, increases in bovine and goat brucellosis contributed to 15% and 33% of the increase in the incidence of new cases of human brucellosis, respectively [7]. Since brucellosis is a zoonosis, its transmission

to humans generally occurs from an animal reservoir [8]. In addition, the ecology of *Brucella* has been changing, and brucellosis surveillance requires new programs for monitoring unusual hosts and ecosystems. For example, in Egypt during 2010, *B. melitensis* was identified in Nile catfish (*Malapterurus electricus*) as a result the dumping of animal waste in the Nile River Delta [9]. These practices are common worldwide; therefore, it is essential to monitor the presence of this pathogen in exposed ecosystems due to the risk of contamination with animal wastes. The gold standard for the diagnosis of brucellosis remains the isolation of *Brucella* spp. bacteria from clinical, environmental, dairy, and derivative samples. However, PCR-based methods that identify nucleic acid fragments from the bacteria are more useful and practical. Therefore, the objective of this study was to determine the presence of *Brucella* spp. on tilapia (*Oreochromis niloticus*) and surface water from a volcanic lake by the application of traditional culture and multiplex PCR methods.

## Materials and Methods

### Bacteria Strains

In the bacteriological and molecular analysis, the reference strains *Brucella suis* 1330 (source ATCC 23444) [10] and *Brucella abortus* 2308, a pathogenic strain from a fetus cow (source Lab collection) [11], were used as controls.

### Fish and Water Sampling

The sampling season was during 2016–2017. It started in May 2016 (spring) with 45 surface water (water recollected at ~15 cm deep) samples recollected from 15 specific points and 15 tilapia fish swab samples obtained across five different sites on the lake (Supplemental Fig. 1). The same number of samples mentioned above from both surface water and fish specimens were collected in August 2016 (summer), at the beginning of December 2016 (fall), and in February 2017 (winter). The specimens, which were adults of tilapia of commercial size (*Oreochromis niloticus*), were collected with the help of local fishermen using traditional fishing methods from San Pedro Lake at geographic coordinates of 21° 12' 33" north latitude and 104° 43' 37" west longitude (Supplemental Fig. 1) and an elevation of 1261 m above sea level within a catchment area of 2.96 km<sup>2</sup> belonging to a priority hydrological region called the Crater Lakes of Nayarit, Mexico [12, 13]. All procedures performed in this study using tilapia were in accordance with the ethical standards of CONAPESCA (responsible for fishing in bodies of continental freshwater waters of the federal jurisdiction of the United Mexican States) and based on the Standard Mexican

Regulation NOM-060-SAG/PESC-2014 [14]. Fishes were transported individually in sterile plastic bags, and surface water samples were recollected and transported to the laboratory at 4 °C and processed within the next 6 h after being collected, according to the American Public Health Association [15]. The geographical position of the sampling points was determined with a Global Positioning System (GPS, GARMIN® eTrex Legend® H), and HOBO Water Level Data Logger was used to monitor the water temperature (°C) of the lake.

### Isolation

A volume of 100 mL of water was filtered in duplicate in a vacuum multiple filtration system (ADVANTEC); the pore size of the filter membrane was 0.22 µm. The first isolation grew in BRUCELLABUAP™ supplemented with Cycloheximide (100 µg/mL), Nystatin (100 U/mL), Nalidixic acid (5 U/mL), and Ethyl violet (0.1% aqueous solution) 1.25 mL/L. The above was modified from Laboratory techniques in brucellosis Manual [16]. Skin swabs were gently rubbed onto the fish surface with a dry sterile cotton-wool swab stick in a manner similar to that used for the filters recovered from each sample and were placed in the culture medium mentioned above, and surface extension was performed on a plate. They were incubated under 5% CO<sub>2</sub> conditions at 37 °C, verifying bacterial growth at 48 and 72 h.

### Bacteriological Analysis

BRUCELLABUAP™, without dyes or antibiotics, was used to grow colonies, which presented the typical morphology of *Brucella* genus bacteria. *Brucella* colonies on clear culture medium are small, transparent, raised, convex, with an entire edge and a smooth, shiny surface. They appear a pale honey color by transmitted light [17]. The bacteria were incubated for 48 to 72 h in 5% CO<sub>2</sub> atmosphere at 37 °C, followed by Gram staining of colonies. Gram-negative colonies with coccobacillary morphology and color were subjected to biochemical testing in Simmons Citrate medium (SCM), Triple Sugar Iron Agar (TSI), Methyl Red–Vogues Proskauer (RM-VP), Lysine Iron Agar (LIA), Christensen Urea, Mobility, Indole, Ornithine (MIO), and catalase and oxidase tests. Subsequently, typing was performed with dyes in BRUCELLABUAP™ culture medium with thionine (1:25,000), thionine (1:50,000), safranin (1:10,000), and fuchsin (1:50,000) [18]. For the abovementioned dilutions, stock solutions were prepared at 0.5% for safranin and 0.2% for thionine and fuchsin (mass/volume). Bacterial suspensions were made in saline solution and adjusted to an optical density of 1.4 to 600 nm. The agar plates with the corresponding dilutions of the dyes were inoculated according to Laboratory techniques in brucellosis Manual [16]. As controls, we used the

reference strains *Brucella abortus* strain 2308, and *Brucella suis* 1330. The plates were incubated at 35 °C with 5% CO<sub>2</sub> and monitored at 48 and 72 h.

### Bacterial Genomic DNA Isolation and PCR Assay

Genomic DNA extraction and purification were performed with a Quick DNA™ Fungal/Bacterial Miniprep Kit from Zymo Research. DNA integrity was confirmed in 1.5% agarose gel, while the purity was determined by spectrophotometer NanoDrop 2000, Thermo SCIENTIFIC<sup>MR</sup>. The reaction mix for Multiplex PCR (mPCR) assay for the differentiation and identification of presumptive *Brucella* isolates from water and tilapia skin and reference strains consisted of GoTaq® Green Master Mix (2×) from Promega (Ref M7122) 12.5 µL and DNA template 1.5 µL (250 ng), with a final volume for each reaction of 25 µL with nuclease-free water based on Promega's specifications. The primers contained in reaction one amplified fragments 774, 587, 450, 272, and 152 bp in size and were used at a concentration of 0.4 µM each. Reaction two contained the primers to amplify fragments 1682, 1071, and 215 bp in size, with a final concentration of 0.4 µM each.

The mPCR was run on a miniPCR™ thermocycler, and the cycle conditions used were one cycle for initial denaturation at 95°C for 3 min, following by 30 cycles for denaturation at 95°C for 1 min, annealing of the primers at 50 °C for 45 s and extension at 72 °C for 3 min, and finally by 1 cycle for final extension at 72°C for 6 min.

The primers used in this assay are shown in Table 1. The mPCR products were analyzed on 1.3% agarose gel. The amplified products were observed on a MultiDoc-It™ System Imaging System – UVP. The multiplex PCR profiles of the reference strains and sample problems were compared with the Bruce-ladder v2.0 multiplex PCR [19, 20]. In Table 2, we show the reported fragments present in each *Brucella* species. Additionally, we included a 215-bp fragment that recently has been suggested to be specific to *Brucella abortus* [21].

### Results

Water and fish samples were collected seasonally for one year and were processed to isolate and identify *Brucella spp.* Seventy-four suspect colonies were isolated by bacteriologic methods and dyes (Table 3). However, of those suspicious colonies, by a multiplex PCR assay. Two colonies isolated from one fish were confirmed to be *B. abortus* and *B. suis* and one colony from surface water sample as *B. abortus*.

The results of the biochemical capacities of the reference strains and *Brucella* isolates were generally similar and are presented in Table 4.

The results of the typing by dyes are shown in Table 5. In general, the presumptive *Brucella* colonies isolated on the BRUCELLABUAP™ by biochemical capacities display the biochemical profile of the *Brucella* genus. The PCR profile of *B. abortus* has been previously reported and is distinguished from the rest of the profiles by the absence

**Table 1** Primers used in this study

| Primer name | Primers sequence (5'3')     | Fragment size (bp) | DNA target  | Reference    |
|-------------|-----------------------------|--------------------|---|--------------|
| BMEI0998f   | ATC CTA TTG CCC CGA TAA GG  | 1682               | Glycosyltransferase, gene <i>wboA</i>   | [19, 22, 23] |
| BMEI0997r   | GCT TCG CAT TTT CAC TGT AGC |                    |   |              |
| BMEII0843f  | TTT ACA CAG GCA ATC CAG CA  | 1071               | Outer membrane protein, gene <i>omp31</i>                                       | [19, 24]     |
| BMEII0844r  | GCG TCC AGT TGT TGT TGA TG  |                    |   |              |
| BME774f     | TCG TCG GTG GAC TGG ATC AC  | 774                | Region fragment <i>wbkF-wbkD</i>  | [19, 25]     |
| BME774rr    | ATG GTC CGC AAG GTG CTT TT  |                    |   |              |
| BMEII0428f  | GCC GCT ATT ATG TGG ACT GG  | 587                | Erythritol catabolism, gene <i>eryC</i> (D-erythrose-1-phosphate dehydrogenase) | [19, 26]     |
| BMEII0428r  | AAT GAC TTC ACG GTC GTT CG  |                    |   |              |
| BMEI0535f   | GCG CAT TCT TCG GTT ATG AA  | 450                | Immunodominant antigen, gene <i>bp26</i>  | [19, 27]     |
| BMEI0536r   | CGC AGG CGA AAA CAG CTA TAA |                    |   |              |
| BR0953f     | GGA ACA CTA CGC CAC CTT GT  | 272                | ABC transporter binding protein   | [28, 29]     |
| BR0953r     | GAT GGA GCA AAC GCT GAA G   |                    |   |              |
| Bru ab2f    | TGA GCG GTG ACA GCC AAG G   | 215                | Autotransporter-associated beta-strand repeat-containing protein                | [21]         |
| Bru ab2r    | GCC AGA ACC CAG CTT CAG A   |                    |   |              |
| BMEII0987f  | CGC AGA CAG TGA CCA TCA AA  | 152                | Transcriptional regulator, CRP family   | [19, 30]     |
| BMEII0987r  | GTA TTC AGC CCC CGT TAC CT  |                    |   |              |

All primers were synthesized by UNIPARTS Inc

**Table 2** Bruce-ladder v2.0 multiplex PCR profile

| Primer name | Fragment size (bp) | <i>B. melitensis</i> | <i>B. ovis</i> | <i>B. abortus</i> | <i>B. suis</i> | <i>B. canis</i> | <i>B. neotomae</i> | <i>B. ceti</i> ** | <i>B. pinnipediae</i> ** | <i>B. microti</i> * | <i>B. inopinata</i> | Reference |
|-------------|--------------------|----------------------|----------------|-------------------|----------------|-----------------|--------------------|-------------------|--------------------------|---------------------|---------------------|-----------|
| BMEI0998f   | BMEI0997r          | 1682                 | +              | +                 | +              | +               | +                  | +                 | +                        | +                   | +                   | [19, 20]  |
| BMEI0843f   | BMEI0844r          | 1071                 | +              | -                 | +              | +               | +                  | +                 | +                        | +                   | -                   | [19, 20]  |
| BMEI1426f   | BMEI1427r          | 774                  | +              | +                 | +              | +               | +                  | +                 | +                        | +                   | +                   | [19]      |
| BMEI0428f   | BMEI0428r          | 587                  | +              | +                 | +              | +               | +                  | +                 | +                        | +                   | +                   | [19, 20]  |
| BMEI0535f   | BMEI0536r          | 450                  | +              | +                 | +              | +               | +                  | +                 | +                        | +                   | +                   | [19, 20]  |
| BR0953f     | BR0953r            | 272                  | -              | -                 | +              | +               | +                  | -                 | -                        | +                   | +                   | [19, 20]  |
| Bru ab2f    | Bru ab2r           | 215                  | -              | +                 | -              | -               | -                  | NR                | NR                       | NR                  | NR                  | [21]      |
| BMEI0987f   | BMEI0987r          | 152                  | +              | +                 | +              | -               | -                  | +                 | +                        | +                   | +                   | [19, 20]  |

All primers were synthesized by UNIPARTS Inc

NR No reported, \*\*extra band between 1071 and 1682 bp, \*extra band between 450 and 587 bp

of the 1071- and 272-bp fragments (Table 2) [31]. The Bruce-ladder v2.0 multiplex PCR of the colonies isolated is shown in Fig. 1. As a positive control, we used a reference strain *B. abortus* 2308, which has an identical profile to other *B. abortus* strains reported [19, 20], although in this work, the profile is shown in Fig. 1a and b. To confirm these results, we used specific primers that amplified a 215-bp fragment with Bru ab2 primers, which are not part of the original Bruce-ladder v2.0 multiplex PCR but are specific to *B. abortus* species, as reported by Alamian et al. (Fig. 1b, lines 2, 3, and 4) [21]. A skin sample from one tilapia fish captured in the fall showed presumptive colonies of *Brucella* spp., which were later confirmed to be two strain, *B. suis* and *B. abortus* afterwards. The multiplex PCR with the DNA from the primary isolate amplified all the fragments reported by the Bruce-ladder v2.0 multiplex PCR. However, the PCR profile did not correspond to any of the *Brucella* species [19, 20] (data not shown) because the DNA of the primary isolate was a mix of both species identified later. Therefore, single colonies were picked and struck to new BRUCCELLABUAP™ to isolate and repeat the Bruce-ladder v2.0 multiplex PCR. The multiplex PCR profile from the isolated colonies showed colonies with specific profiles of *B. abortus* and *B. suis*, respectively (Fig. 1). It is worth mentioning that in the reaction mix for panel A (Fig. 1), the primers used were BMEI0987 (152pb), BR0953 (272 pb), BMEI0535–BMEI0536 (450 pb), BMEI0428 (587 pb), and BMEI1426–BMEI1427 (774 pb). In the reaction mix for panel B (Fig. 1), the primers used were Bru ab2 (215 pb), BMEI0843–BMEI0844 (1,071 pb), and BMEI0998–BMEI0997 (1,682 pb).

## Discussion

Brucellosis remains as a major zoonosis worldwide, and according to the World Animal Health Information Database, Mexico had the largest number of reported outbreaks of brucellosis—5514 in 2014 [32]. Nayarit is a state of Mexico that is in the eradication phase of brucellosis. However, brucellosis cases are still presented in the State [33, 34]. San Pedro Lake belongs to a hydrological region called the Crater Lakes of Nayarit, Mexico [13].

One sample from surface water isolated in summer was positive for *B. abortus*, as confirmed by the Bruce-ladder v2.0 multiplex PCR (Fig. 1) and colonies isolated from fish skin; in fall, the PCR profile showed colonies with specific profiles of *B. abortus* and *B. suis*. To the best of our knowledge, this is the first report of two *Brucella* species isolated from the skin of tilapia (*Oreochromis niloticus*). Previously, *B. melitensis* species were isolated from 120 catfish captured from Nile canals and 120 farmed catfish from skin, spleen, liver, and kidney [9]. The authors

**Table 3** Colonies isolated and identified by bacteriological analysis

| Date            | Season    | Morphologically positive strains isolated for <i>Brucella</i> spp. |           | Positive strains by biochemical tests and dyes |           | *Location site from positive strain by biochemical test and dyes |
|-----------------|-----------|--|-----------|--|-----------|--|
|                 |           | Surface water  | Skin fish | Surface water                                  | Skin fish |  |
| Month/day/year  | 2016–2017 |  |           |  |           |  |
| May/6/2016      | Spring    | 16   | 5         | 0  | 0         |  |
| August/20/2016  | Summer    | 12   | 22        | 1  | 0         | 6  |
| December/7/2016 | Fall      | 9  | 5         | 0  | 2         | 2  |
| February/4/2017 | winter    | 3  | 2         | 0  | 0         |  |

\*Supplementary Figure S1. Sampling points

**Table 4** Biochemical capacities for *Brucella* isolates, *Brucella suis*, and *Brucella abortus* 2308

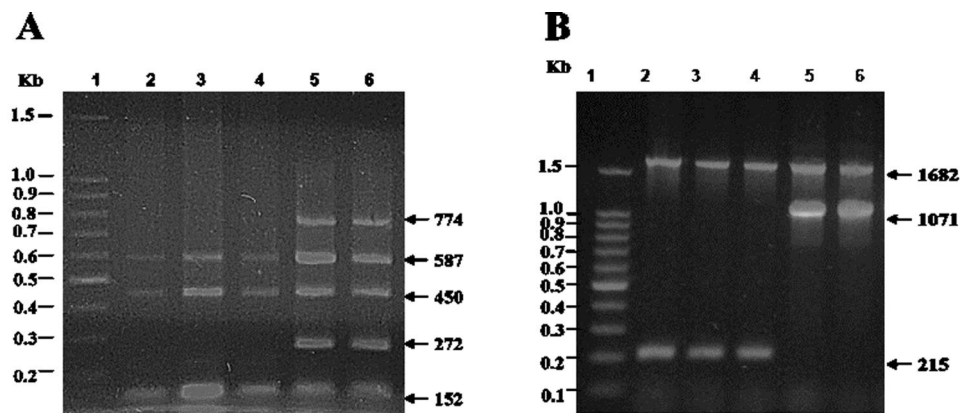
| Biochemical capacities | <i>B. abortus</i> (2308) | <i>Brucella suis</i> (1330) | <i>Brucella</i> spp. from skin fish | <i>Brucella</i> spp. from surface water |
|------------------------|--------------------------|-----------------------------|-------------------------------------|---|
| Gram stain             | Gram-negative            | Gram-negative               | Gram-negative                       | Gram-negative                           |
| Morphology             | Coccobacilli             | Coccobacilli                | Coccobacilli                        | Coccobacilli                            |
| CO <sub>2</sub>        | Positive                 | Slow growth                 | Slow growth                         | Positive                                |
| SCM                    | Negative                 | Negative                    | Negative                            | Negative                                |
| TSI                    | Negative                 | Negative                    | Negative                            | Negative                                |
| RM                     | Negative                 | Negative                    | Negative                            | Negative                                |
| VP                     | Negative                 | Negative                    | Negative                            | Negative                                |
| LIA                    | Negative                 | Negative                    | Negative                            | Negative                                |
| Christensen urea       | Positive (++)            | Positive (+++)              | Positive (++)                       | Positive (++)                           |
| Mobility               | Negative                 | Negative                    | Negative                            | Negative                                |
| Indole                 | Negative                 | Negative                    | Negative                            | Negative                                |
| Ornithine              | Negative                 | Negative                    | Negative                            | Negative                                |
| Catalase               | Positive                 | Positive                    | Positive                            | Positive                                |
| Oxidase                | Positive                 | Positive                    | Positive                            | Positive                                |

**Table 5** Typing by sensitivity to dyes for bacteria of the genus *Brucella*

| Dyes                 | <i>B. abortus</i> strain 2308 | <i>B. suis</i> strain 1330 | <i>Brucella</i> spp. from skin fish | <i>Brucella</i> spp. from surface water |
|----------------------|-------------------------------|----------------------------|-------------------------------------|---|
| Thionine (1:50,000)  | Negative                      | Positive                   | Positive                            | Negative                                |
| Thionine (1: 25,000) | Negative                      | Positive                   | Positive                            | Negative                                |
| Fuchsin (1:50,000)   | Positive                      | Negative                   | Positive                            | Positive                                |
| Safranin (1:10,000)  | Positive                      | Negative                   | Positive                            | Positive                                |

suggested that Nile catfish are naturally infected by contaminated materials from infected ruminants that reach the water canals and that direct contact with infected catfish may also be a source of human brucellosis [9]. Some of the activities in the Nile River Delta region include agriculture and livestock [35], which are similar to those carried out in San Pedro Lake. At the north of Lake San Pedro, where we isolated the *Brucella* species, the mouth of the San Pedro stream and the San Pedro Lagunillas town are located and tourist and fishing activities are carried out.

In the south of the lake, livestock activities such as grazing cattle and agriculture are carried out around the lake, similar to the activities of the Cuenca Oriental, Mexico. In a previous work, carried out in the crater lakes from the Cuenca Oriental, Mexico, we isolated *Brucella* spp. from internal organs from charales (*Poblana*) [36]. It has been reported that *Brucella* spp. can survive in tap water at  $-4$  °C for 114 days, 57 days at 8 °C and pH 6.5 in lake water [37], but in the presence of organic matter, such as cattle feces, soil, and lake water at 37 °C, they can survive



**Fig. 1** Bruce-ladder v2.0 multiplex PCR agarose gel picture used to confirm the *Brucella* species isolated. **a** PCR profile showing fragments of 152, 272, 450, 587, and 774. Lane 1, molecular weight marker 100-bp DNA Ladder (PROMEGA), lane 2, *Brucella* isolate from surface water (*B. abortus*), lane 3, *Brucella* isolate from skin fish (*B. abortus*), lane 4, PCR profile from *B. abortus* 2308 (positive control). Lanes 5 and 6, *Brucella* isolate from skin fish (*B. suis*) and *B. suis* 1330 (ATCC 23,444) positive control, respectively, which is different from the other profiles above. **b** PCR profile showing frag-

ments of 215, 1071, and 1682 bp. The 1682- and 1071-bp bands were weakly revealed in panel A) PCR; therefore, they were amplified in a separate PCR with a 215-bp fragment, which is specific for *B. abortus*. Lane 1, 100-bp molecular weight marker; lane 2, PCR profile of *Brucella* isolate from surface water (*B. abortus*); lane 3, Fish skin *Brucella* isolate (*B. abortus*); lane 4, PCR profile *B. abortus* 2308 (positive control); lanes 5, PCR profile isolated from fish skin *Brucella* (*B. suis*); and lane 6, *B. suis* 1330 (ATCC 23,444) positive control

for more than 2 years [38]. However, in lake water with a temperature of 37 °C and a pH 7.5, the bacterial survival is less than one day [37]. In the case of the volcanic lake of this study, the average water temperatures in summer and fall were  $27.03 \pm 0.46$  °C and  $20.04 \pm 0.22$  °C, respectively. Probably given the water and environmental conditions, it allowed *Brucella* to survive and to be isolate in this study. Although *Brucella* has been observed to survive for some time in open environments, the bacterium hardly divides and eventually dies [39]. However, there are no studies about *Brucella* species survival on fish skin, which could be a source of infection to humans. The isolated strains probably came from breeding animals common in the region (Supplemental Fig. 1), such as pigs or cattle that could be infected with *Brucella abortus* or *B. suis*, the latter of which is derived from the breeding and grazing of cattle that takes place on the periphery and the shore of the lake (Supplemental Fig. 2B and C). When the sampling was carried out, it was possible to observe that some cattle entered the lake (Supplemental Fig. 2A), and given the water temperature, the organic matter present in the volcanic lake and the rainfall, which carry animal wastes from pastureland and urban settings, the water may be the source of contamination. In addition, the San Pedro stream passes near cattle and pig raising pens and urban areas before reaching the volcanic lake (Supplemental Fig. 1), where pollutants such as fecal matter from domestic and human livestock can run off relatively easy and quickly into surface waters and contaminate the volcanic lake and the fish that live there.

**Author Contribution** LdCRR: Conceived and designed the analysis, collected the data, contributed data or analysis tools, performed the analysis, and wrote the paper. ZSA: Conceived and designed the analysis, collected the data, contributed data or analysis tools, performed the analysis, and wrote the paper. LML: Conceived and designed the analysis, collected the data, contributed data or analysis tools and article review. YML: Contributed data or analysis tools and article review. EICR: Conceived and designed the analysis, collected the data, contributed data or analysis tools, performed the analysis, and wrote the paper.

**Funding** This study was funded by Consejo Nacional de Ciencia y Tecnología, México (Grant Number INFR-2012-2:188090).

## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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