Circulating strains of *Brucella abortus* in cattle in Santo Domingo de los Tsáchilas Province – Ecuador

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**INTRODUCTION**

Brucellosis is a widespread zoonotic disease, affecting cattle, sheep, goats, pigs, and humans (1). From a total of nine species of *Brucella* reported so far, four species are zoonoses: *Brucella abortus*, *B. canis*, *B. melitensis*, and *B. suis* which have been typically related to cattle, dogs, sheep goats, and pigs, respectively. Other species such as *B. microti*, *B. neotomae*, *B. ovis*, *B. pinnipedialis*, and *B. inopinata* are supposed to be host specific (2, 3).

In cattle, the main symptoms associated with brucellosis include abortion and poor health in newborn calves. Epididymitis and infertility have been also reported in bulls (4, 5). In Ecuador, annual losses due to brucellosis in cattle are estimated to be around 5.5 million USD due to abortions, reduced milk yield, and mortality (6). In addition, in several municipalities in Ecuador, the presence of brucellosis in humans has been directly related to its presence in the cattle population (7), with, so far, only, *B. abortus* as the causative agent of human brucellosis (8, 9), contrary to neighboring Colombia and Peru, were in addition to *B. abortus*, *B. melitensis*, and *B. suis* have equally been reported in man (8, 10).

Determining the strain variability of *Brucella* can be helpful to understand the geographical and epidemiological dispersion of the disease as shown in the United States where molecular techniques have been used to evaluate strain diversity of *B. abortus* to define foci of transmission between cattle and wildlife, i.e., elk and bison, and also to identify infections related to the use of vaccines (11). In northern Ecuador, previous studies have reported *B. abortus* biotype 1 and 4 in human samples (9, 12), yet the diversity of *Brucella* sp. in cattle has not been investigated previously.

The livestock market in Santo Domingo de los Tsáchilas province is the largest in the country because of its strategic geographical location (13). This cattle market is very informal, facilitating the movement and exchange of animals and meat to large cities. It is also an important center for the trade of animals from the dairy areas of the Sierra region to different areas in the coastal region for fattening bull calves, as such it is hardly surprising that many of the outbreaks of foot-and-mouth disease started in this region (13). Thus, the sanitary condition of animals in this region might offer a reflection of the health status of cattle from different zones of the country. In this context, and given the zoonotic risk related to cattle brucellosis, the evaluation of the disease prevalence supported by a study of strain variability in cattle...
passing through this region will be an important epidemiological tool, including the assessment of the importance of food-borne brucellosis.

MATERIALS AND METHODS

STUDY DESIGN

The study area was located at Santo Domingo de los Tsáchilas Province (0.14°: −0.70°N, −78.73°: −79.62°E). In total, 656 blood samples were collected from 12 sero-positive dairy farms, previously identified during a large-scale national survey (data not published) and at the provincial abattoir between May and June 2013. Samples were analyzed by Rose Bengal plate (RB) and Wright's Slow Agglutination Test with EDTA (SAT-EDTA). Equally, milk and supra-mammary lymph nodes were carefully sampled avoiding contamination and stored at 4°C until screening by RB and/or SAT-EDTA. Samples from positive reactors were processed for bacterial growth in the specific growth medium.

SEROLOGICAL TESTS

All blood samples were tested by Rose Bengal (Bengatest antigen® 4% v/v suspension) and Wright's SAT-EDTA (antigen SAW®, Synbiotics ASAW code). For RB, the slightest trace of agglutination was considered as positive. For SAT-EDTA, 100 µl of antigen was added to a doubling serum dilution from 1/12.5 up to 1/25.600. Data were recorded as international agglutination units (international units per milliliter) with values equal or greater than 30 IU/ml, corresponding to a transparency of 25% of a 1/25 dilution, considered as a positive reactions as described by Godfroid and Boelaert (14).

MICROBIOLOGICAL ISOLATION

In a microbiology laboratory (biosafety type III), lymph nodes were macerated using the Stomacher®, lymph nodes were centrifuged at 3000 g for 10 min. Both macerated nodes and cream were tested for bacterial growth in selective Farrell medium [Columbia blood agar base CM0331 (Oxoid) + horse serum (reference: 16050-130 Gibco) + modified Brucella Selective Supplement SR0083A (Oxoid)]. Cultures were kept at 37°C and 5% CO2 for 5 days (15). Then, isolates were transferred to agar base [Columbia blood agar base CM0331 (Oxoid)] to obtain distinct Brucella sp. colonies. Finally, part of the colonies was used for DNA extraction and another part was stored at −70°C for further analysis.

BIOTYPING AND MOLECULAR IDENTIFICATION

Isolated colonies were biotyped by macroscopic observation and biochemical assays, i.e., urease, catalase, oxidase, and hydrogen sulfide production. Additionally, bacterial cultures were grown on media with stained safranin, thionin, and fuchsin at different concentrations, and tested for agglutination with Anti-A and Anti-M mono-specific sera (15).

For molecular identification, genomic DNA was extracted according to Marmur and Kirby [phenol–chloroform–isoamyl alcohol (16)]. DNA amplification was performed using protocols IS711-PCR and AMOS-PCR as described by Ref. (17, 18) to identify genera and species, respectively. Primers for DNA amplification are presented in Table 1. Each PCR-reaction had a final volume of 20 µL. Master mix was made with 1 U/45 µl of Taq Polymerase, 1X buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 mM

Table 1 | Primers used in the study.

| Primer (name) | 5′-3′ Sequence |
|---------------|----------------|
| Primer sequence for IS711-PCR for genus identification | | |
| IS6501 3′ | GAT AGA AGG CTT GAA GCT TGC GGA C |
| IS6501 5′ | ACG CGG GTG TAT GGG AAA GGC TTT T |
| Primer sequence for conventional AMOS-PCR for species identification | | |
| B. abortus-specific primer | GAC GAA CGG AAT TTT TCC AAT CCC |
| B. melitensis-specific primer | AAA TCG CGT CCT TGCTG TCT GA |
| B. ovis-specific primer | CGG GTT CTG GCA CCA TCG TCG |
| B. suis-specific primer | CGG CGG TTT TCT GAA GGT TCA GG |
| IS711-specific primer | TGC CGA TCA CTT AAG GGC CTT CAT |
| Primer sequence for “HOOF-prints” biotype | | |
| Locus-1 | GGT GAT TGC CGG GTG GTT CCG TTG AAT GAG |
| Locus-2 | CCC GCA TGA TCC GCG AAC AGC TGG ATG |
| Locus-3 | CAG CGG CTT GAG GAT GAG GCG GCA G |
| Locus-4 | GCA GAA TTT TCG AGG CAT TCG GCG ATG |
| Locus-5 | GTG CTC CAG GGC GCC GGG AGG ATG TTT TAG |
| Locus-6 | GCC GCA GGA AAG CAG CGG ATC TGG AGAT TTA TC |
| Locus-7 | CAG AGC CGT CCG TGG TTA CTT GAG TAG GGC AG |
| Locus-8 | GTG GGA ACG GTT ATC CTT TAA CGG GAG TAA GGG |
| REV-1 | GGG GAG TAT GTT GTT GGT CAT GAC CGC |
| REV-3 | GGG GGC ART ARG GCA GTA GTG TAA GGG AAT AGG G |

*R = A to G.
of each primer, and approximately 10 ng of DNA. To characterize the *Brucella* biotype, the “HOOF-Print” technique was used as described by Bricker et al. (19) and Bricker and Ewalt (20) for eight loci; all VNTR were amplified separately using primers described in **Table 1**; each PCR-reaction had a final volume of 15 µl and the master mix was composed with 0.6 U of Taq Polymerase, 1X buffer, 1.5 mM MgCl₂, 0.25 mM dNTPs, 0.2 mM of each primer, and approximately 10 ng of DNA.

**DATA ANALYSIS**
The proportions of isolation of *Brucella* sp. were contrasted by Fisher exact test with 5% statistical significance. Additionally, an estimation of the test concordance was measured in terms of positive and negative agreements over the total isolations. Data were analyzed in “R” software version 3.1.0.

**RESULTS**
**SEROLOGY**
Out of 656 blood samples, 50 were sero-positive, i.e., 25 were from the slaughterhouse and 25 were from sero-positive dairy farms of Santo Domingo.

**MICROBIOLOGICAL ISOLATION**
Twenty-five milk and 25 lymph node samples were processed and isolated in a specific microbiological medium. The bacterial growth of *Brucella* spp. was evidenced in nine (36%) and four cases (16%), respectively. No statistical difference was found between the types of sample used for the isolation (p-value = 0.1085); yet isolation from milk appeared to be better than from tissues.

**BIO-TYPIFICATION**
**Table 2** shows the biochemical features of the microbiological isolations from sero-positive animals and from those where *Brucella* was isolated (milk or supra-mammary lymph nodes). Out of nine milk isolations, six were biochemically compatible with *B. abortus* biotype and three were “not determined” isolations (ND, samples: 8, 10, and 13) because they did not present urease activity, nor growth in CO₂ and no H₂S production. Isolations from lymphatic nodes (samples 1–4) were also biochemically compatible with *B. abortus*. In total, nine isolates were sensitive to inhibition by basic fuchsin, four were insensitive but agglutinated with anti-A sera. Nine isolates agglutinated with anti-A sera (i.e., samples 1–5, 6, 7, 9, and sample 11) and only one agglutinated with anti-M sera (sample 12) hence corresponding to

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**Table 2** | Differential characters of *B. abortus* and biotypes isolated from milk and lymph nodes collected in Santo Domingo de los Tsáchilas province.

| Sample no. | Code | Source | Activity | Growth on dye media | Agglutination in mono-specific sera | Biotypes |
|------------|------|--------|----------|---------------------|-----------------------------------|----------|
|            |      |        | Oxidase  | Catalase | Urease | CO₂ | H₂S | Fuchsin | Safranin | Thionin 20 µg | Anti-A | Anti-M |
| 1          | 1482 | Lymph node | +       | +       | +       | +   | +   | − | − | − | + | − | Bvar1 |
| 2          | 1483 | Lymph node | +       | +       | +       | +   | +   | − | − | − | + | − | Bvar1 |
| 3          | 1550 | Lymph node | +       | +       | +       | +   | +   | − | − | − | + | − | Bvar1 |
| 4          | 1552 | Lymph node | +       | +       | +       | +   | +   | − | − | − | + | − | Bvar1 |
| 5          | 1476 | Milk | +       | +       | +       | +   | +   | − | − | − | + | − | Bvar1 |
| 6          | 1285 | Milk | +       | +       | +       | +   | +   | − | − | − | + | − | Bvar2 |
| 7          | 1286 | Milk | +       | +       | +       | +   | +   | − | − | − | + | − | Bvar2 |
| 8          | 1294 | Milk | +       | +       | −       | −   | −   | − | + | + | − | − | ND |
| 9          | 1301 | Milk | +       | +       | +       | −   | −   | − | − | − | + | − | Bvar2 |
| 10         | 1302 | Milk | +       | +       | +       | −   | −   | − | − | − | + | − | Bvar2 |
| 11         | 1303 | Milk | +       | +       | +       | −   | −   | − | − | − | + | − | Bvar2 |
| 12         | 1307 | Milk | +       | +       | +       | +   | +   | − | − | − | + | − | Bvar4 |
| 13         | 1308 | Milk | +       | +       | +       | +   | +   | − | − | − | + | − | ND |

*ND, not determined.*

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**Table 3** | Genotyping of *Brucella* spp. from isolates of milk and lymph nodes collected in Santo Domingo de los Tsáchilas province.

| Sample no. | Code | PCR²-IS711 | AMOS³-PCR | VNTR⁴ |
|------------|------|-------------|------------|--------|
| 1          | 1482 | +          | B. abortus | Bvar1 |
| 2          | 1483 | +          | B. abortus | Bvar1 |
| 3          | 1550 | +          | B. abortus | Bvar1 |
| 4          | 1552 | +          | B. abortus | Bvar1 |
| 5          | 1476 | +          | B. abortus | Bvar1 |
| 6          | 1285 | +          | B. abortus | Bvar1 |
| 7          | 1286 | +          | B. abortus | Bvar1 |
| 8          | 1294 | +          | B. abortus | Bvar1 |
| 9          | 1301 | +          | B. abortus | Bvar1 |
| 10         | 1302 | +          | B. abortus | Bvar1 |
| 11         | 1303 | +          | B. abortus | Bvar1 |
| 12         | 1307 | +          | B. abortus | Bvar4 |
| 13         | 1308 | +          | B. abortus | Bvar1 |

²PCR, polymerase chain reaction.
³AMOS-PCR, PCR for detection of *B. abortus, B. melitensis, B. ovis, and B. suis*.
⁴VNTR, variable number of tandem repeat.
Table 4 | HOOF-Prints: results of alleles configuration to identify Brucella abortus biotypes from isolates of milk collected in Santo Domingo Province in Ecuador.

| Sample | Code | Locus-1 | Locus-2 | Locus-3 | Locus-4 | Locus-5 | Locus-6 | Locus-7 | Locus-8 | Biotype |
|--------|------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| 6      | 1285 | 4       | 3       | 6       | 6       | 5       | 6       | 3       | 2       | Bvar1   |
| 7      | 1286 | 4       | 3       | 6       | 5       | 6       | 6       | 3       | 2       | Bvar1   |
| 9      | 1301 | 4       | 3       | 6       | 6       | 5       | 6       | 3       | 2       | Bvar1   |
| 11     | 1306 | 4       | 3       | 6       | 6       | 5       | 6       | 3       | 2       | Bvar1   |
| 12     | 1307 | 7       | 4       | 5       | 3       | 2       | 2       | 7       | 2       | Bv4     |

*Samples shown in this table correspond to samples that were different from B. abortus Bvar1 in biotyping; i.e., Biotype 2 and 4.

B. abortus biotype 4. As described by Corbel and Brinley Morgan (21), Mayfield et al. (22), and Rodriguez Torres et al. (23), growth in basic fuchsin medium and agglutination with anti-A sera, is indicative for B. abortus biotype 1; however, lack of bacterial growth in basic fuchsin and agglutination with anti-A sera is indicative for B. abortus biotype 2. Yet, as shown in Table 2, by molecular analysis, all isolates were B. abortus biotype 1. All milk isolates were identified as B. abortus biotypes 1 and 4.

MOLECULAR IDENTIFICATION

In total, 13 isolates corresponded to B. abortus identified by IS711 and AMOS-PCR (Table 3). The “HOOF-Prints” protocol allows biotype classification, as such VNTR markers evidenced the presence of B. abortus biotype 1 in 12 out of 13 isolates. All these isolates were field strains and were different from vaccine strains S19 and RB51, as confirmed by conventional AMOS-PCR. Furthermore, one isolate, from a milk sample, was confirmed to be B. abortus biotype 4 (Sample 12). The allelic diversity found in Brucella isolates from Santo Domingo Province is given in Table 4. Molecular patterns found are similar to biotype 1 and 4, reported by Bricker et al. (19). Samples, biochemically found as biotype 2 (samples 6, 7, 9, and 11), were confirmed as B. abortus biotype 1 whilst sample 12 was corroborated as biotype 4.

On the other hand, the concordance of biochemical and molecular tests estimated a proportion of coincidences of 76.92%.

DISCUSSION

This study demonstrated the presence of bovine brucellosis in the province of Santo Domingo de los Tsáchilas province.

Biochemical tests used for biotyping isolates allowed the identification of B. abortus biotypes 1, 2, and 4, biotypes which have been previously reported in humans in Ecuador using biochemical and molecular techniques (9, 12). Samples 6, 7, 9, and 11, were biochemically identified as B. abortus biotype 2, yet as B. abortus biotype 1 by HOOF-Prints protocol, which is highly sensitive test (11, 19). It is known that the biochemical tests are of limited use for identifying biotypes, since the biochemical response depends on environmental conditions during the preparation of media and reagents and the amount and time for growth of the strains (24–26). In addition, the intraspecific Brucella molecular variability could have caused this biochemical response (21–23, 27). However, further studies are suggested to confirm or reject the presence of B. abortus biotype 2 in Ecuador or that the biochemical results are due to a genetic adaptation of B. abortus biovar 1.

Molecular tests indicated that all strains described in this study were field strains and not vaccine-type strains; as for B. abortus biotype 1 field strains, in spite of being genetically similar to vaccine strains, the former do not grow in thionin (2 μg/ml) in a culture medium.

The presence of B. abortus biotype 4 as previously reported in humans by Ron-Román et al. (9), was confirmed in this study. The biochemical characteristics of B. abortus biotype 4 differ from B. abortus biotype 1 and 2 because the former is agglutinated by anti-A instead of anti-M sera. In the same way, the allelic configuration allowed differentiating between biotypes 1 and 4 in HOOF-Prints technics.

The type strains of all classical Brucella species and biovars were surveyed to assess the discriminating power of microsatellite fingerprint technique. This technique was used to assess the level of divergence amongst and within populations of naturally infected cattle and wildlife (19, 20, 28, 29).

In this survey, both B. abortus biotype 1 and 4 were reported as described by Ron-Román et al. (9, 12) in humans from northern Ecuador. The presence of the two biotypes (1 and 4) in animals in Santo Domingo province shows that due to intensive cattle movement, the presence of several biotypes is possible. Finally, the study findings suggest that microbiological isolation of Brucella spp. is more successful from milk samples (44%) than from lymph nodes in slaughter cattle (16%).

In conclusion, the strain diversity of B. abortus was assessed in a region with intensive cattle movement and B. abortus biotypes 1 and 4 were found; although, some isolations of B. abortus biotype 1 presented phenotypic variability according to biochemical tests. These findings were correlated with results found in humans in northern Ecuador. Further research is needed to study intra-species variability and to investigate the possibility of other biotypes and Brucella species present in the tropical regions of Ecuador.

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