The unexpected discovery of *Brucella abortus* Buck 19 vaccine in goats from Ecuador underlines the importance of biosecurity measures

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Abstract

Very few, mostly old and only preliminary serological studies of brucellosis in goats exist in Ecuador. In order to assess the current epidemiological situation, we performed a cross-sectional serological study in the goat populations of Carchi (n=160 animals), Pichincha (n=224 animals), and Loja provinces (n=2,024 animals). Only two positive serological results (RB negative and SAT-EDTA ≥400 IU/ml) were obtained in lactating goats from the same farm in Quito (Pichincha province). Additionally, milk was sampled from 220 animals in Pichincha province. The present study indicates a low apparent prevalence in Pichincha province and absence in Carchi and Loja provinces. A total of 25 positive milk ring tests (MRT) were obtained in Pichincha province yielding a prevalence of MRT of 11.16%. Subsequent culture was performed on the positive MRT samples. All results were negative, apart from a single sample, obtained from a serological positive goat in Quito, that was positive for Brucella abortus strain 19 (B19). Several hypotheses are forwarded concerning this unexpected result. The most likely hypothesis is the possible accidental use of a needle, previously used for vaccination of cattle with the said vaccine, for the administration of drug treatment to the goat. This hypothesis underlines the necessity of biosecurity measures to prevent this type of accidents.
Introduction

Brucellosis is a worldwide disease with health and economic impacts (Castro et al., 2005). It is widely distributed in humans and animals, especially in developing countries. Its occurrence is related to the existence of animal reservoirs and high infection rates in livestock, especially in goats and sheep (Corbel, 2006).

The main cause of caprine brucellosis is *Brucella melitensis* (biovars 1, 2 and 3) (Godfroid et al., 2010) but some sporadic cases caused by *B. abortus* are documented (e.g., Leal-Klevezas et al., 2000). One or more of the following typically characterize the clinical form of the disease: abortion, retained placenta, orchitis, epididymitis and, more rarely, arthritis together with excretion of the organisms in uterine discharges and milk (OIE, 2016a).

Surveillance in goats by indirect diagnostic methods is not a common practice in most countries of South America (PANAFTOSA, 2000), where goat breeding is constrained in its development, because of conditions of overcrowding, poor or non-existent disease control measures and lack of technical assistance, which, together with rudimentary empirical management, permit the transmission of brucellosis (Ortega-Sánchez et al., 2009).

Caprine brucellosis due to *Brucella melitensis* is present in Mexico, Peru, Argentina, Paraguay and Bolivia (Aznar et al., 2014; PANAFTOSA, 2000). Until now, there are no reports in Ecuador of isolation and characterization of *Brucella melitensis* in bovines or goats, only molecular findings that demonstrate its presence in samples of lymphatic nodes from goats at the slaughterhouse of Quito (Luna et al., 2016) The total number of goats is estimated between 178,000 (INEC et al., 2002) and 191,000 (OIE, 2016b) of which approximately 43% (78,000) are found in the canton of Zapotillo in Loja province.
The marketing of goat milk in different parts of the Metropolitan District of Quito (two million inhabitants) has become a common activity and forms the basic income of several families engaged in this business. Ecuadorian law prohibits peddling unpasteurized milk, and although vendors work without government regulation, they try as much as possible to maintain minimum health standards, such as collecting animal droppings, washing the udder and selling milk in new and clean bottles (El Comercio, 2012).

The very few serological studies of brucellosis in goats conducted in Ecuador are old and incomplete or preliminary (e.g., Poulsen et al., 2014). In order to determine the seroprevalence of *Brucella* spp. in goats in three selected areas of Ecuador, as well as isolate the causative agent, we conducted a cross-sectional study (serum and milk samples) in Carchi, Pichincha and Loja provinces.

Materials and methods

Selected areas

The selection of three areas for this study is based on the potential risks: Bolivar and Mira cantons of Carchi province (presence of bovine brucellosis in cattle and existence of mixed farms) (Ron-Román et al. unpublished data), the urban and peri-urban Metropolitan District of Quito in Pichincha province (business of milk goats in Quito city and high density of inhabitants) and Zapotillo canton of Loja (high density of goats) provinces (Figure 1).

Sampling design

A survey with census sampling at farm level (n=86) and convenience sampling at animal levels (n=2,408) was performed in the three selected areas. In Carchi and
Pichincha provinces (small herds), all herds and all animals present in a herd were sampled. In Zapotillo canton of Loja province (large herds), all herds were included and a random selection of 25% of animals present in a herd was sampled.

In Carchi, blood was sampled between December 2012 and February 2013 (n = 160 goats in 12 herds). In urban and peri-urban Quito (Pichincha province), blood and milk were sampled between December 2009 and April 2010 (n = 224 and 220 goats in 12 herds for blood and milk samples, respectively). In Zapotillo canton of Loja province, blood were sampled in July 2011 (n = 2,024 goats in 62 herds). The milk samples were collected only in Quito, area with positive results to serology, to perform the isolation and characterization of the pathogen.

**Samples**

The goats sampled belonged to native, Nubian and Anglo-Nubian breeds. Jugular vein blood was sampled in vacutainer tubes (10 ml). Each sample was centrifuged; the serum was identified, analysed, and stored at -20°C. In addition, 100 ml of milk was collected from each lactating goat sampled in peri-urban Quito. All milk samples were identified, stored in a cool box until analysis at the Instituto de Investigación en Salud Pública y Zoonosis (CIZ, Central University of Ecuador).

**Blood and milk analysis**

Serum samples were analysed for the presence of antibodies against *Brucella* spp. using two diagnostic tests: slide agglutination test with Rose Bengal (RB) and the serum agglutination tube test with EDTA (SAT-EDTA). These tests were performed as previously described (Alton et al., 1988; OIE, 2016a). The modified MRT test as described by Mancera and Ontiveros (2001) for diagnose of brucellosis in goats, was
performed as a complementary test on the milk samples. The modification consisted in the addition of 0.3ml of a NaCl solution [25%] and 0.1ml of corn oil to each milk sample (1ml). Afterwards, the samples were incubated at 37°C for 2 hours.

Isolation and identification of Brucella spp.

Milk samples from SAT-EDTA positive (n=2) and MRT positive animals (n=23) were centrifuged at 2,000 g for 15 minutes. The supernatant (cream) and sediment were grown in selective Farrell medium (Columbia Agar Base [Oxoid CM0331] with 5 % decomplemented horse serum [GIBCO Ref-16050-130] and Brucella selective supplement [OXOID SR0083A]) for the isolation of Brucella spp.

Replicated colonies with BASE medium (Columbia Agar Base with 5 % decomplemented horse serum) were identified and classified by means of: macroscopic and microscopic observation, Gram staining and oxidase [DIFCO-BBL Ref: 261181], catalase and urease tests. The procedures were performed as previously described (Alton et al., 1988; Godfroid and Boelaert, 1995).

Identification and molecular characterization of Brucella spp.

Once identified by biochemical tests, the Brucella colonies were analysed molecularly by three different PCR tests: the IS6501 PCR or PCR-IS711 (primers: IS6501 3 ': 5'-gat-aga-agg--gct-gaa ctt tgc-gga-c-3' IS6501 5': 5'-acg-ccg-gtg-tat-ggg-aaa-ggc-ttt-t-3') for genus identification, AMOS PCR (Primers: B. abortus-specific: gac-gaa-egg-aat-ttt-tcc-aat-ccc; B. melitensis-specific: aaa-tcg-cgt-cct-tgc-tgg-tct-ga; B. ovis-specific: cgg-gtt-ctg-gca-cca-tcg-tcg; B. suis-specific: cgc-cgg-ttt-tct-gaa-ggt-tca-gg; IS711-specific: tgc-cga-tca-ctt-aag-ggc-ctt-cat) (Bricker and Halling, 1994) for species determination and modified AMOS PCR (Primers: RB51/2308: ccc-cgg-aag-ata-tgc-ttc-

**Statistical analysis**

The seroprevalence was estimated with a Binomial exact distribution and computed in Stata/MP 14.1 (StataCorp, 2015).

**Results**

No serological RB test showed the presence of antibodies in any of the animals tested but some animals originating from Pichincha province (see below) tested positive for the SAT-EDTA.

The study demonstrated the absence of antibodies to *Brucella* spp in Bolivar and Mira cantons of Carchi province (Number of animals tested [Nt]=160; seroprevalence of 0 % with 95 % confidence interval [CI]:0-1.85 %) and Zapotillo canton of Loja province (Nt=2.024; seroprevalence of 0 % with 95 % CI=0-0.15 %). The seroprevalence of brucellosis in the district of Quito in Pichincha province was quite low (Nt=224; seroprevalence of 0.89 % with 95 % CI=0.11-3.19 %).

Of the 220 MRT that were performed in Pichincha province, 25 were positive (milk prevalence of 11.16 % with 95 % CI=7.35-16.03 %). Only two goats (out of 47 originating from the same farm in the Tiwinsa sector, urban Quito) were positive in SAT-EDTA (high antibody titres) and in MRT (Table 1). From the two seropositive and lactating goats from Quito urban area, one *Brucella* was isolated on milk. This strain was future characterized and identified as *Brucella abortus* strain 19. The results of the microbiological characterization are in Table 2. A fragment of 498 bp, specific for *Brucella abortus*
biotypes 1, 2 or 4, according to Bricker and Halling, (1994), is shown in Figure 2. In Figure 3, the absence of the 364 bp fragment (tandem IS711) and the *eri* fragment of 178 bp, demonstrate that the strain found in the goat is the B19 vaccine strain (Bricker and Halling, 1995). A further 23 lactating goats that were positive in MRT were negative in culture.

**Discussion**

Brucellosis is a contagious infectious disease, caused by bacteria of the genus *Brucella* spp., which affects both human and several animal species. Caprine brucellosis is mainly due to *B. melitensis* (Godfroid et al., 2010) and some cases of *B. abortus* was previously published (e.g., Leal-Klevezas et al., 2000). The pathogenicity in humans for these two species of *Brucella* is high (Godfroid et al., 2010; Saegerman et al., 2010).

The use of SAT-EDTA, RB and MRT was previously evaluated for the diagnosis of caprine brucellosis (Falade, 1978). There was a good correlation between SAT-EDTA and RB when both tests were negative but RB failed to detect 80% of sera above 50 IU/ml in SAT-EDTA. Also, owing to the relatively poor milking potential of the goat and the false positive results with MRT, it was concluded that the SAT-EDTA offers a better serological diagnostic tool for caprine brucellosis. This study is in line with this previous information. Unfortunately, studies reporting serological test results in goats should be interpreted with caution, as most of the data have been obtained without isolation of *Brucella* (Mancera and Ontiveros, 2001).

Several preliminary results are available in some Faculties of Veterinary Medicine in Ecuador. In Guayas province (west central part of Ecuador), 33 % of 800 individual milk samples were positive to MRT in 1970 but with no isolation of *Brucella* (Albornoz, 1970). Three other serological studies with Huddleson agglutination test in Macará (Granda,
1972), Loja (Tapia, 1998) and Azuay (Sánchez, 1997) provinces indicated a zero or very low seroprevalence.

The present study indicates a low prevalence in Pichincha province and absence in Carchi and Loja provinces.

The discovery of the *B. abortus* strain 19 (B19) in milk from a goat with a positive serology result (SAW-EDTA: 3,200 IU/ml; high IgM level) was unexpected. Several hypotheses can be postulated. The first hypothesis is the improper use of brucellosis B19 vaccine in goats in addition to its advised use in cattle. The brucellosis vaccine of choice for goats is Rev 1 and, as recommended, B19 is only mandatory in cattle in Ecuador and common in Pichincha province. The second hypothesis is a use of a needle, which was previously used for B19 vaccination in cattle, for the administration of a drug to goats.

Goats and other species present in a herd are commonly treated by drug injection with the same needle. The second serologically positive goat comes from the same herd, which may form an indication of possible serial use of the same needle. The third hypothesis is the consumption of milk by goats originating from B19 vaccinated cattle. Positive microbiological cultures were obtained during a period of three years from the milk of cows vaccinated with B19 (Meyer and Nelson, 1969), as well as in colostrum (Corner and Alton, 1981). Seropositive titres were observed for a period of one year after B19 vaccination of cows (Manthei, 1952). A study of oral vaccination with B19 showed the need of a large dose (500 billion cells) and all serological test were negative in heifers 82 days after vaccination (Nicoletti and Milward, 1983). Despite the fact that it cannot be excluded, this hypothesis is deemed unrealistic. The fourth hypothesis is the excretion of B19 in the environment by vaccinated bovines and the use of a same pasture by goats. The intermittent excretion of B19 strain was detected by PCR until 9 years in vaccinated cattle mainly in urine and also in milk samples, which confirmed its multiplication and
persistence (Pacheco et al., 2012). However, in this study cultures were always negative. For identical reasons (large dose needed and short period of positivity in serological tests) this hypothesis also appears improbable. In conclusion, the second hypothesis is retained as the most likely.

Conclusion

The study demonstrated the absence of antibodies to Brucella spp in Bolivar and Mira cantons of Carchi province and Zapotillo canton of Loja province, the principal goat producing canton. Isolation of Brucella abortus strain 19 in a goat in Quito district demonstrates the possible cross-infection from vaccinated cattle (B19 vaccination is common here), probably through the accidental use of a needle previously used for vaccination of cattle with B19 vaccine. This finding highlights the necessity of stringent biosecurity measures and quality control of vaccination campaigns.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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Figure 1: Goat population per Canton and localization of the study areas (INEC et al., 2002)

Legend: [A], Bolivar and Mira cantons of Carchi province (presence of bovine brucellosis in cattle and existence of mixed farms); [B], urban and peri-urban Metropolitan District of Quito in Pichincha province (business of milk goats in Quito city and high density of inhabitants); [C], Zapotillo canton of Loja province (high density of goats).

Figure 2: PCR amplification products from *Brucella* strains tested by the conventional AMOS assay

Legend: MP: Molecular weight marker; B1, B2, B3 and B4: Samples of *Brucella* strains by bovines; C1: Samples of *Brucella* strains by caprine (amplification of IS711 which is specific for *B. abortus* biovars 1, 2 or 4 [498 bp]); C-: negative control; C+: positive control of *B. abortus* biovar 1.

Figure 3: PCR amplification products from *B. abortus* strains tested by the modified AMOS assay.

Legend: MP: Molecular weight marker; B1, B2, B3 and B4: Samples of *B. abortus* strains by bovines; C1: Samples of *Brucella* strains by caprine (absence of amplification of tandem *IS711* [364 bp] and *eri* locus [178 bp]); C-: negative control; C+: positive control of *B. abortus* biovar 1.
Table 1. Serology, culture and polymerase chain reaction (PCR) results of two SAT EDTA positive goats

<table>
<thead>
<tr>
<th>Sample N°</th>
<th>Herd Code</th>
<th>Province</th>
<th>Canton</th>
<th>Method of diagnostic</th>
</tr>
</thead>
<tbody>
<tr>
<td>178</td>
<td>Tiw 3</td>
<td>Pichincha</td>
<td>Quito</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SAT-EDTA 400 IUÁ</td>
</tr>
<tr>
<td>184</td>
<td>Tiw 3</td>
<td>Pichincha</td>
<td>Quito</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SAT-EDTA 3200 IUÁ</td>
</tr>
</tbody>
</table>

Legend: RB, Rose Bengal test; SAT – EDTA, Serum agglutination test with EDTA; MRT, Milk Ring Test IUA, International Units of Agglutination PCR-IS711, Polymerase chain reaction with insertion 711; AMOS PCR, Abortus, Melitensis, Ovis and Suis; mAMOS PCR, AMOS modified (PCR for the differentiation of vaccine strains from field strains).
Table 2. Characterization of the caprine *Brucella* spp. isolate

<table>
<thead>
<tr>
<th>Bacteriological sample code</th>
<th>Catalase</th>
<th>Oxidase</th>
<th>Urease activity</th>
<th>CO₂ requirement</th>
<th>CO₂ production</th>
<th>H₂S production</th>
<th>Thionin 20 µg</th>
<th>Thionin 10 µg</th>
<th>Basic Fuschin 20 µg</th>
<th>Safranin 100 µg</th>
<th>anti A</th>
<th>anti M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ec-CIZ-Cap-1</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B2*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B9**</td>
<td>+</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>+</td>
</tr>
<tr>
<td>B1***</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

Legend: EC-CIZ-Cap-1 is the caprine *Brucella* isolate; * control *Brucella abortus* biovar 2; ** control *Brucella abortus* biovar 9; *** control *Brucella abortus* biovar 1; a positive for most strains.
Fig. 2

B. abortus specific
Fig. 3

<table>
<thead>
<tr>
<th>MP</th>
<th>B1</th>
<th>B2</th>
<th>C1</th>
<th>B3</th>
<th>B4</th>
<th>C-</th>
</tr>
</thead>
</table>

⇐ Tandem IS711

⇐ *eri* locus