Human brucellosis in North-West Ecuador: typifying of *Brucella* spp., sero-prevalence, and associated risk factors

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Running title: R**isk factors for human brucellosis in Ecuador**

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Abstract

Human brucellosis in Ecuador is underreported and based only on passive surveillance. Since 2008, brucellosis was removed from the list of communicable diseases in the country. Until now, the true human brucellosis picture has not yet been determined. The aim of this study was to determine the seroprevalence of the disease, to identify risk factors associated with brucellosis seropositivity in humans and to isolate circulating strains of *Brucella* spp. in the north-western part of Ecuador.

Between 2006 and 2008, a large transect survey was conducted, based on blood sampling of people from the north-western part of Ecuador (N=3,733) together with an epidemiological inquiry. Based on three diagnostic tests used in parallel, the overall seroprevalence was estimated as 1.88% (95% C.I.: 1.48-2.38). Based on a multivariable random effects logistic regression analysis, the main risk factors associated with human brucellosis seropositivity were: contact with livestock (OR = 3.0; C.I.: 1.25 – 7.08), consumption of foetus and placenta (OR = 2.5; C.I.: 1.18 – 5.22) and involvement in activities at risk for brucellosis infection (OR =1.8; C.I.: 1.00 – 3.35). Noticeable variation in brucellosis seropositivity among humans within cantons was observed. The circulating strain was *Brucella abortus* biotype 4.

The study emphasized that contact with livestock, consumption of foetus and placenta and occupational hazard group were all significant risk factors for the transmission of brucellosis among individuals in the north-western part of Ecuador. Alongside encouraging the launching of educational campaigns against brucellosis, especially in rural areas where 36% of the population lives, controlling this zoonotic disease in animals will directly benefit its prevention in humans especially since there is no safe and efficacious vaccine against brucellosis in humans.

Keywords: Brucellosis; Human; Ecuador; Serological tests; True prevalence; Risk factors; *Brucella abortus* biotype 4

Introduction

Brucellosis is an infectious and contagious disease caused by Gram-negative coccobacilli, which can survive in the cells of the immune system. It has a high tendency to cause chronic infections both in humans and in cattle (Young 2007, Moriyon 2001, Torres at al. 2004, Saegerman 2010).

In many countries, brucellosis is an important disease that causes serious economic losses in cattle production (FAO 2003, Guillén 2006, WHO 2006). In Ecuador these losses are estimated at 5.5 million US$ per year (Torres 2008). In humans, this zoonosis mainly leads to loses in working time and costs related to diagnosis and treatment (Bowden 1996).

In Latin America, four in ten people live in areas where brucellosis is endemic in natural animal reservoirs (Alvarez 2001). However, the infection in humans is underestimated and often not reported (Dean et al., 2012) and only few reports exist concerning the identification of circulating strains of *Brucella* spp. (e.g. Deodato et al., 2011 ; Aznar et al., 2012; Ron-Román et al., 2012). In addition, the true incidence of this zoonosis has not yet been estimated (Lucero et al 2008; Aznar et al., 2012).

In Ecuador, by means of diagnostic assays with low sensitivity, several authors have reported the presence of antibodies against *Brucella* spp. mainly among slaughterhouse workers: Intriago (1971) reported a prevalence of 4% (1/25), León (1979 cited by Delgado 1992) detected 10.90% (23/211), Zurita (1980, cited by Díaz 2001) detected

23.83% (56/235) and finally Delgado (1992) mentioned a seroprevalence of 2.32% (4/173).

Despite brucellosis being a communicable disease in Ecuador since 2007, the true incidence of human cases remains largely unknown. According to the Ministry of Health (MSP), only 111 human cases were reported between 1990 and 2007 (EPI-2 2008), whereas, the National Institute for Statistics and Census (INEC) registered 152 persons hospitalised for brucellosis between 1995 and 2007 (INEC 2008).

The aim of the present work was to obtain a realistic figure of the prevalence of human brucellosis by determining the seroprevalence of antibodies against *Brucella* spp., and by identifying the causal agent together with possible infection-associated risk factors among people living and/or working in the north-western part of Ecuador.

Materials and methods

Description of the study and selection of the study region

Between 2006 and 2008, a transect study was conducted, based on blood sampling of people from the north-western part of Ecuador together with an epidemiological survey. After informed consents were obtained, blood samples were taken from persons inhabiting the high-altitude or Sierra provinces such as Carchi, eastern Imbabura, eastern Pichincha and the coastal provinces such as Esmeraldas, Manabí, western Imbabura and western Pichincha.

Selection of provinces was based on the high provincial-level seroprevalence of bovine brucellosis reported in Ecuador. Seroprevalence was officially estimated to be between 4.0% and 10.62% in the Sierra and between 5.88% and 10.62% in the Coast (Torres 2008, MAG-SESA 1999). Prior to this study, the seroprevalence was also estimated to be between 2.17% and 9.42% using the Rose Bengal Test (RB) and indirect Enzyme

Linked Immunosorbent Assay (iELISA), respectively in bovines of Santo Domingo (Pichincha) and between 1.08 and 9.73% to RB and iELISA, respectively in El Carmen (Manabí) (Angula and Tufino, 2005). The selection of the study zone was also based on the occurrence of 41.30% (19/46) of the human cases, as reported by MSP between 1997 and 2007 (EPI-2 2008) and 51.97% (79/152) of the hospitalized brucellosis patients, as reported by INEC (2008). In addition, since sheep, goats and camel populations are very small in the study area, only the link between brucellosis seroprevalence in bovines and humans was investigated. A map of the study area is shown on Figure 1.

Samples

After informed consent, a total of 3,733 blood samples were taken from people with different occupational hazards. A first possible high-risk group of people (n=2,444) consisted of labourers at cattle farms, slaughterhouse workers, meat and organ traders, cattle traders, veterinarians, zoo workers, teachers and students from a faculty of animal production and farm and slaughterhouse managers. A second possible low-risk group of people (n=1,289) consisted of agricultural labourers, informal traders, public servants, school and college teachers and students, house workers and transporters.

Alongside collecting blood samples, other information was collected through personal face-to-face interviews. The questionnaire recorded the following information for each subject: age, sex, consumption of raw milk (yes or no), consumption of blood (yes or no), consumption of cheese (yes or no), region (Sierra or tropical), consumption of foetus or placenta (yes or no), occupational hazard group, contact with animals (yes or

no) and presence of symptoms such as pyrexia, weakness, sweating, muscle pain, backache, and headache suggestive of brucellosis (Mantur et al. 2006).

Based on results of the questionnaire the people were divided into two groups, *i.e*. those working directly or indirectly in a slaughterhouse (n=542) and those who were not (n=3,191). The full questionnaire is available upon request from the corresponding author.

Diagnostic assays

Three serological assays to detect antibodies against *Brucella* spp. were used: Rose Bengal fast agglutination test (RBT), Wright's Slow Agglutination Test with EDTA (SAT-EDTA) and indirect Enzyme Linked Immunosorbent Assay (iELISA). Samples were processed and analysed in the laboratory for immunodiagnosis at the International Centre for Zoonoses (CIZ) of the Central University of Ecuador (UCE).

Rose Bengal fast agglutination test (RB)

The RB assay was used with Bengatest® antigen i.e. a concentrated suspension (4% v/v) of *B. abortus* Weybridge strain 19, heat and phenol (0.5%) inactivated, suspended in an acid buffer and stained with Rose Bengal. Equal quantities (30 µl) of serum and antigen were mixed in a well (4 min) on a glass plate and any degree of agglutination was considered a positive reaction.

Wright's Slow Agglutination Test with EDTA (SAT-EDTA)

For SAT-EDTA, the antigen (Antigen SAW®, Synbiotics code # ASAW) was a concentrated suspension of *B. abortus* (strain 1119/3), heat and phenol (0.5%) inactivated and suspended in a phenol-buffer at 0.5%. The assay was performed as

described by Godfroid & Boelaert, (1995) with serum dilutions of 1/12.5, 1/25, 1/50, 1/100, 1/200, 1/400, 1/800, 1/1,600, 1/3,200, 1/6,400, 1/12,800 and 1/25,600 in a constant volume (100 µl) of antigen. Quantitative results were given as International Units of Agglutination (IU/ml). A value equal to or above 100 IU/ml, corresponding to 75% transparency of dilution 1/50), was considered as a positive reaction.

Indirect Enzyme Linked Immunosorbent Assay (iELISA)

The assay was performed according to Godfroid & Boelaert (1995). Smooth *B. abortus* Weybridge strain 19 lipopolysaccharide (LPS) antigen was incubated on polystyrene plates for 3.5 h at 37°C and overnight at 4ºC. Plates were washed 5 times with a washing solution (NaCl 0.9% + Tween 20 at 0.01%).

Then, 50 µl of 1/50 diluted serum in glycine-EDTA-Tween 80 buffer (BB) was added per well and the calibration curve was determined at dilutions 1/270, 1/540, 1/1,080, 1/2,160, 1/4,320, 1/8,640. After one hour incubation at ambient temperature, the solutions were discarded, plates were washed 5 times and 50 µl conjugate (Protein G-HRPO, Pirce CD47675, diluted at 1/1,500 in G - HRPO + FCS at 2%) was added to each well and left to incubate at ambient temperature for 1 hour.

The same washing procedure was repeated and 100 µl substrate solution (i.e. *o*-PD Ortho-phenylendiamine tablets, SIGMA P-8287, one tablet of 10 mg dissolved in 25 ml citrate phosphate buffer SIGMA P-4809 + 5 µl H_2O_2 at 30%) was added to each well. Plates were left to incubate for 20 min in the dark at ambient temperature. Subsequently the reaction was stopped by adding 25 µl H_2SO_4 (2M) to each well. Optical densities (OD) were read by a spectrophotometer (STAT FX 2100), with filters between 492nm and 630nm. Mean OD values of the samples and the calibration curve were corrected by subtracting the mean BB from the mean OD.

A cut off value, above which a sample was considered positive was set at or above 20 units (U)/ml. This cut-off value was established based on local epidemiological conditions and to optimize the compromise between sensitivity and specificity (Franco et al., 2007; Gómez et al., 2008; Soudbakhsh et al., 2009).

Calculation of the units was based on the reference values of the curve i.e. 1.87 U, 3.75 U, 7.5 U, 15 U, 30 U, and 60 U, for dilutions 1/270, 1/540, 1/1,080, 1/2,160, 1/4,320, 1/8,640 respectively.

***Isolation and typing of Brucella* spp.** (according to Alton et al. (1988))

Due to the lack of standardized procedures in Ecuador, the isolation of the causal agent was based on blood cultures - BACTEC (3 repetitions with 30 minutes-intervals) from persons with high serotitres (n=22) (Yagupsky, 1994; Cetin et al., 2007). Blood cultures were done at the "Hospital Vozandes Quito", where they were maintained for 30 days, and after those days the cultures were considered as negatives.

Isolated *Brucella* were identified and typified by CIZ and also by Veterinary and Agrochemical Research Centre (VAR-CERVA) as a reference laboratory using (1) macroscopic and microscopic observation of the colonies in cultures, (2) biochemical assays (oxydase, catalase and urease), (3) production of H_2S, (4) CO_2 growth requirement, (5) growth in stained media (thionine, basic fuchsin, safranin), (6) agglutination with mono-specific sera A and M, and (7) PCR – AMOS as described by Bricker and Halling (1994).

Statistical analysis

To determine the potential risk factors associated with human brucellosis sero-positivity, a two stage modelling approach was used. In this approach, individuals were

considered positive if they tested positive in at least one serological test along with the presence of any of the clinical symptoms suggestive of brucellosis as previously mentioned.

Firstly, a univariate analysis was performed using a random effects logistic regression model. The model used as response, the brucellosis status of the individuals (1 for positive and 0 for negative) and each risk factor or indicator variable in turn as the independent variable. The possible effects of variations in brucellosis seropositivity among the different provinces and cantons were accounted for by incorporating province and canton as a random effects in the model (VanLeeuwen et al. 2010).

Secondly, variables with a p-value ≤ 0.25 in the univariate analysis were further analysed in a multivariable random effects logistic regression model. A manual forward stepwise model building approach was employed with the Akaike's Information Criterion (AIC) as the calibrating parameter to select the final model. The model with the smallest AIC is considered to be the most appropriate model. All two-way interaction terms of the variables remaining in the final model were assessed for significance. The effects of confounding were investigated by observing the change in the estimated coefficients of the variables that remain in the final model once a non-significance variable is included. When the inclusion of a non-significant variable led to a change of more than 25% of any parameter estimate, that variable was considered to be a confounder and was included in the model (Dohoo et al. 2003).

The intra-class correlation coefficient (ICC), which is a measure of the degree of clustering of individuals belonging to the same province and canton, was computed (Snijders and Bosker 1999).

The models were built using the xtmelogit () function in STATA, version 12, software (SataCorp LP, College station, Texas). Model selection was done using Laplacian

223 approximation whereas parameter estimates from the final model were obtained using
224 Adaptive Gaussian Quadrature (Twisck 2003). The robustness of the final model was
225 assessed by increasing the number of Quadrature (integration) points and monitoring
226 changes in parameter estimates (Frankena et al. 2009).
227
228 ***Ethical considerations***
229 The protocol was thoroughly reviewed and approved for ethics by the Bioethics
230 Committee of the Biomedical Center, Central University of Ecuador. Prior to being
231 included in the study, all participants provided informed written consent. For minors,
232 parents/guardians provided consent on their behalf.
233
234 **Results**
235 ***Descriptive statistics***
236 A total of 3,733 persons, with a mean age of 30.03 ± 16.26 years (min=3, max=89
237 years) were sampled in five provinces in the north-western part of Ecuador: Carchi,
238 Imbabura, Esmeraldas, Manabí and Pichincha. Seventy people with mean age 37.86 ±
239 14.81 years (min=10, max=79 years) reacted positive to at least one of the three
240 diagnostic tests, representing an overall sero-prevalence of 1.88% (C.I. 1.48 -2.38). The
241 proportion of seropositive people between groups (slaughterhouse workers *versus* other
242 people) was 4.8% (26/542) and 1.4% (44/3,191), respectively. This suggests a
243 preferential repartition of seropositive people in slaughterhouse workers (Pearson' chi-
244 squared test = 29.4; $P<0.001$).
245 The distribution of the number of individuals tested, the number and percentage of
246 seropositives is presented in Table I. Besides teenagers and children represented 20.84%
247 (778/3,733) of the sample with only six of them being seropositive (3 originating from

248 farms). The information about this sub-population is presented in the Table II. This
249 suggests a preferential repartition of seropositive cases among older people (Pearson'
250 chi-squared test = 6.50; *P*=0.01).
251
252 ***Risk factors for human brucellosis seropositivity based on the univariate random***
253 ***effects logistic regression analysis***
254 Based the results of the univariate random effects logistic regression analysis with
255 random intercepts for both province and canton, the factors; age, sex, contact with
256 livestock, contact with foetal secretions, consumption of foetus and placenta and
257 involvement in activities at risk were all statistically significantly associated with
258 human brucellosis seropositivity ($p < 0.05$) (Table III). On the other hand consumption
259 of raw cow milk and consumption of fresh blood were not significant at the 5% level
260 but since their p-values were ≤ 0.25, they were considered as potential risk factors and
261 were thus included in the multivariate random effects logistic regression analysis.
262
263 ***Final model based on multivariate random effects logistic regression analysis***
264 Out of the potential risk factors initially considered in the multivariate random effects
265 logistic regression model, 3 were included in the final model (*i.e.*, **consumption of**
266 **foetus and placenta, contact with livestock and** occupational hazard group).
267 In addition, the results were not confounded by any of the variables not included in the
268 final model. Increasing the number of quadrature points had no influence on the
269 estimated fixed effects and the variance component parameters indicating that the model
270 is robust. The estimated odds ratios and their 95% confidence intervals are presented in
271 Table IV. There was no variability in brucellosis seropositivity among provinces but
272 rather a higher variability among people within provinces.

Typifying of circulating Brucella spp. *in North-West Ecuador*

Detailed information about persons with positive blood cultures (n=22) is given in Table V with the characteristics of the isolates, bacteriological data and PCR in Table VI and Figure 2, respectively. From three positive cases, *Brucella abortus* biotype 4 was isolated and typified. Blood cultures were only positive for patients with higher levels of IgM antibodies (SAT-EDTA) suggesting an acute brucellosis.

Retrospectively, seropositive persons (N = 70) were queried about possible symptoms related to brucellosis, a summary of the outcome based on the questionnaire is presented in Table VII.

Discussion

The current study aimed to provide a reliable estimation of the sero-prevalence based on the detection of antibodies against *Brucella* spp., the isolation and the identification of the circulating strain of *Brucella* spp. and the identification of possible risk factors related to the transmission and spread of brucellosis among people in the north-western part of Ecuador.

Prevalence of human brucellosis in the north-western part of Ecuador

In the current study an overall sero-prevalence of 1.88% (C.I. 1.48 - 2.38) was found, which may be in sharp contrast with the official data of the Ecuadorian Ministry of Health MSP i.e. only 67 cases between 2003 and 2007 (EPI-2 2008). The results of the present investigation, and additional observations described by Ron-Román et al. (2012) in humans, as well as, a seroprevalence of 2% in bovines from the same study area, and numerous isolations of *Brucella* sp. from different animal reservoirs (bovines and

canine) (unpublished data), suggest an underreporting of human brucellosis in Ecuador considering that 36% of the population lives in rural areas (Organización Panamericana de la Salud, 2008).

Based solely on clinical symptoms, a correct diagnosis of brucellosis is not possible (Abdoel & Smits 2006; Saegerman et al. 2010) and even microbiological blood cultures are sometimes unreliable because sensitivity is too variable and too dependent on the stage of infection (*i.e.* acute stage) and the *Brucella* species concerned (Casao et al., 2004). The difficulties related to the diagnosis and the often ambiguous or even absent clinical symptoms, also noted in the present study, are probably the principal reasons for the sub-notification (Serra and Godoy 2000, Agasthya et al. 2007).

The non-existence of a vaccine against brucellosis in humans or the difficulty to access a safe and efficacious vaccine implies that controlling this zoonotic disease in animals will directly benefit its prevention in humans especially to improve the biosecurity. A joint work between the Ecuadorian Ministries of Public Health (MSP) and the Livestock, Aquaculture and Fisheries (MAGAP) is needed to consolidate a "One Health" initiative.

Risk factors for human brucellosis in the north-western part of Ecuador

The occupations that expose people to the infection are male dominated in this study region thus the apparent increased risk for infection. Several other studies have indicated gender as a significant risk factor for brucellosis (Wassif et al. 1992, Shehata et al. 2001, Mantur et al. 2007, Meky et al. 2007). The apparent elevated risk associated with older age groups could be explained simply by the fact that older people had more occasions to contract the disease (Cooper 1992, Kalaajieh 2000). Nevertheless, it is important to mention that 3 cases were also found in children below 15 years old,

confirming the findings of Guevara et al. (Guevara 2009) that children are indeed at risk and also do get the infection, e.g. due to direct or indirect contact with animals when accompanying adults handling livestock (Minas et al. 2007) or through consumption of non-pasteurised dairy products (Issa and Jamal 1999).

Brucellosis is mainly an occupational disease and the multivariate analysis indicated that the odds of brucellosis seropositivity among those working in slaughterhouses were higher than those of people in the general population. This is in line with the results of other studies (Omer et al. 2002, Rahman et al. 2012). The higher sero-prevalence among slaughterhouse workers confirms the proposition that intimate contact with animals is more important than consumption of infected dairy products (McDevitt 1971).

According to WHO (2006), temperatures for pasteurisation or boiling milk should be sufficiently high to eliminate bacteria and to render it fit for consumption. Nevertheless the relation between transmission of brucellosis and raw milk consumption in the present study was not statistically significant which is in line with Serra and Godoy (2000) reporting no link between presence of antibodies against *Brucella* spp and the unhygienic consumption of milk. This lack of an association between consumption of milk or dairy products and infection may also be due to the low number of seropositives people found in our study that consumed these products.

The non-significance of the consumption of cheese squares with findings from Barroso-García et al. (2007) where it was observed that the consumption of cheese is not necessarily a source of infection of brucellosis, because processing takes a few days or even weeks, affecting the number of bacteria, which was also indicated in this study. However, this information depends largely on the maturation process of each cheese considered and thus caution is recommended.

In general, the shedding of *B. abortus* in cows naturally infected is lower (< 10^3 CFU/ml for several weeks but with decreasing after the partum) than for *B. melitensis* in small ruminants (in general > 10^3 CFU/ml during all the lactating period) (Carpenter and Boak 1928, Jouve 1952, Grilló et al. 1997, Hamdy and Amin 2002, Saegerman et al. 2010). In addition the human pathogenicity of *B. abortus* appears lower than *B. melitensis* (Godfroid et al. 2010). These elements are other possible explanations for the lack of evidence found in this study considering the link between consumption of milk and dairy products and brucellosis infection.

Traditionally, milk and dairy consumption without any sanitary measures has been considered the most important route of transmission. However, recent reports stress the prominent role of transmission by direct contact with animal reservoirs (Saegerman et al. 2010, Barroso-Garcia et al. 2007, Godfroid et al. 2010).

In Ecuador, a national program exists. The main objective is to obtain free brucellosis farms on a voluntary basis. In fact, this program is restricted to some farmers which are able to pay for the vaccination of calves with the B19 or RB51 vaccine, to test animals every six months and to eliminate infected animals without compensation (most often at the slaughterhouse). In addition, no control of animal movements is performed and control of dairy farms by MRT is not systematized and suffers from the lack of availability of antigen. However the milk marketed in the cities by companies is usually pasteurized. However, raw milk is sold frequently in rural and peri-urban areas.

This study has not demonstrated the importance of raw milk consumption in the human brucellosis transmission, in Ecuadorian conditions. However serial isolation of *B. abortus* (N ~ 100) from bovine raw milk of the same area (Ron-Román et al., unpublished data) indicates that the risk exists and needs future additional investigation.

Not surprisingly, the consumption of foetus or placentas was significantly associated with brucellosis seropositivity. This alimentary tradition, though largely obsolete, is still commonly used by rural families, and even in public restaurants that offer Ecuadorian typical dishes called foetus (locally known as “ville”) or placentas (locally called “guagua mama - huagra mama”). This meat is cooked but handling of this food item increases risk of exposure to *Brucella* spp.

Unfortunately, the consumption of blood was not significantly associated with brucellosis seropositivity in Ecuadorian context. However, this practice can be at risk but necessitates a donor in the acute phase of brucellosis which is not frequent (Thiange et al. 1992).

Education and health campaigns should target the elimination of such practices. It has indeed been observed in the population of the north-western part of Ecuador that risks related to eating habits are mostly due to a lack of basic knowledge about brucellosis and the modes of transmission.

Typifying of circulating Brucella spp. in North-West Ecuador

Biotyping *Brucella* is important for the epidemiological knowledge, since it can reveal geographical characteristics (FAO and OMS 1986) and allows a better understanding of the spread of the disease (Roux 1979). Unfortunately isolating and typing of *Brucella* spp. is not always possible since it requires high biosecurity laboratories and trained personnel. Furthermore, the low number of successful isolations in the present study is mainly due to the low number of patients with acute brucellosis (*i.e.*, with high levels of IgM antibodies) and also partly due to the localisation of the bacteria in specific tissues and organs like bone marrow, cerebrospinal fluid (CSF), liver, kidneys and spleen, which renders isolation from blood very unlikely (Doganay and Aygen 2003).

In the present study 24.29% of the persons with a positive sero-reaction showed no apparent symptoms at all. This is lower than the 45.6% reported by Pila-Perez et al. (Pila-Perez 1997) and the 99% found in a retrospective study of the symptomatology by Hernández et al. (1999).

Although according to Pappas et al. (Pappas et al. 2006), based only on few reported data, Ecuador was not considered an endemic country for human brucellosis, the present results, based on factual data, contradicted this statement, especially in rural areas where 36% of the population lives. A recent report presented a systematic review of the scientific literature published between 1990 and 2010 relating to the frequency of human brucellosis in the world indicated that underestimation of the disease could be related to barriers in accessing health care or to case mismanagement and misdiagnosis (Dean et al. 2012). In Latin America, according to the previous report, reliable information was found only for Argentina and Mexico at sub-national level.

Conclusions

The absence of a National Policy and differential diagnostic tests hinders the development of surveillance and control programmes in high-risk areas for human brucellosis (especially in rural areas). It is thus difficult to have a realistic idea about the incidence of this disease. In the past, little attention was given to brucellosis in Ecuador and it is necessary to develop programmes to control (and eventually eradicate) brucellosis in the identified risk areas whereby highly sensitive diagnostic methods should be used both for humans and for animals with the objective to obtain an early warning system and to determine the correct prevalence at national level.

In view of the results of this study, there is an urgent need for information campaigns, especially in rural area, about the risks involved following direct contact with livestock

and consumption of foetus and placenta and equally about the preventive care as to avoid infection. Also, more investigations to isolate and identify the biotypes of *Brucella* spp. circulating in Ecuador should be followed.

Finally, it is of utmost importance that evidence-based information could be given to national and international donor organisations involved with future prevention, control and research programmes on brucellosis.

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Conflicts of interest

The authors declare that there are no competing financial interests.

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671 **Figure 1: Location of the study area**

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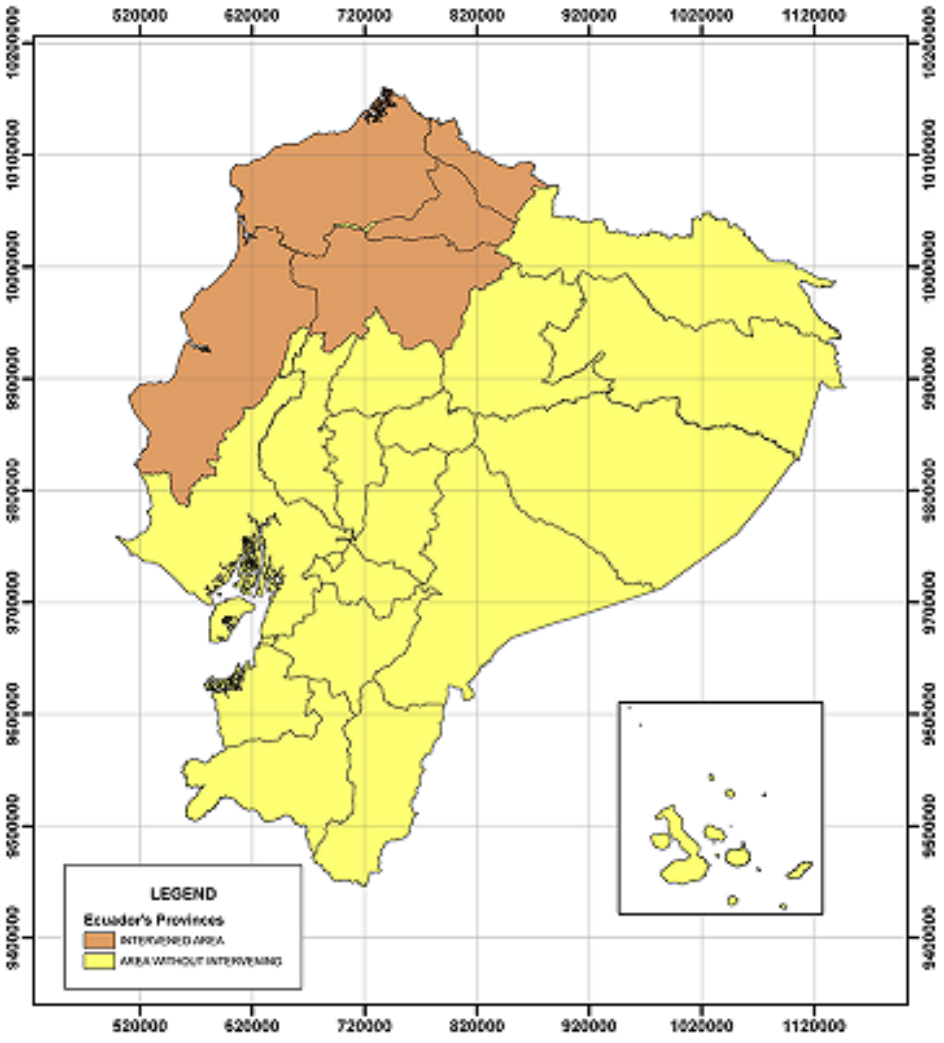

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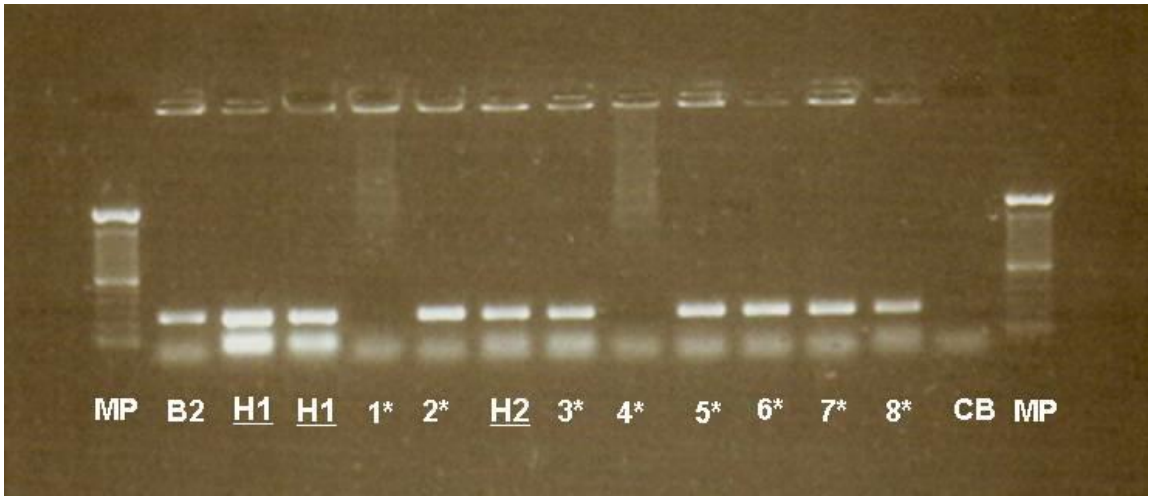
Figure 2. PCR – AMOS of *Brucella* from blood cultures, isolated from positive persons.



Legend: MP: Molecular weight marker, B2: control *Brucella abortus* biotype 2, H1: Human sample 1 (Ec-CIZ-Hum-1), H2: Human sample 2 (Ec-CIZ-Hum-2); *: Samples from complementary studies at CIZ; CB: Blank control.

687 **Table I.** Human brucellosis in North-West Ecuador: results of three diagnostic assays
688

RB	SAT-EDTA	iELISA	SLA	OTH	Number (%)
-	-	-	516	3,147	3,663 (98.12)
-	-	+	2	5	7 (0.19)
-	+	-	0	0	0 (0)
-	+	+	1	1	2 (0.05)
+	-	-	1	10	11 (0.29)
+	-	+	17	14	31 (0.83)
+	+	-	1	0	1 (0.03)
+	+	+	4	14	18 (0.48)
Total			542	3,191	3,733 (100)

689
690 Legend: RB: Rose Bengal; SAT-EDTA: Wright's Slow Agglutination Test with EDTA;
691 iELISA: Enzyme Linked Immunosorbent Assay; SLA: people working in a
692 slaughterhouse; OTH: other people.
693
694

695 **Table II.** Detailed information from six seropositive patients under 18 years old

696

Nº	Province	Canton	Age	Sex	Membership	Contact with livestock	Contact with foetal secretions	Consumption of milk	Consumption of placenta and foetus	Consumption of blood	RB	SAT-EDTA (UI/ml)	iELISA (U/ml)
1	Pichincha	Mejía	10	M	farm	+	-	boiled	-	-	-	50	30
2	Pichincha	Mejía	16	F	farm	+	+	raw	-	-	-	30	30
3	Pichincha	Mejía	17	M	farm	+	-	boiled	-	-	+	80	60
4	Imbabura	Ibarra	11	F	rural community	-	-	raw	-	-	+	-	-
5	Imbabura	Ibarra	10	M	rural community	-	-	boiled	-	-	+	30	-
6	Imbabura	Urcuquí	17	F	rural community	+	+	boiled	-	-	+	30	-

697

698

699 Legend: RB: Rose Bengal; SAT-EDTA: Wright's Slow Agglutination Test; iELISA: indirect Enzyme Linked Immunosorbent Assay.

700 **Table III.** Distribution of seropositive results and potential risk factors for human

701 brucellosis in the north western part of Ecuador

Factor	Tested	Seropositives (%)	*Odds ratio*	95% C. I.	*p*-value
Age (years)					
<=15	681	3 (0.4)	1	ref	0.0252
From 16 to 45	2382	50 (2.1)	4.4	1.3-14.6	
>=46	667	17 (2.5)	4.5	1.3-16.0	
Sex					
Women	1570	22 (1.4)	1	ref	0.0415
Men	2163	48 (2.2)	1.7	1.0-2.9	
Region					
Tropics	1185	16 (1.4)	1	ref	0.1013
Sierra	2548	54 (2.1)	1.580.7	0.89-3.00	
Occupational hazard group*					
Low	3191	44 (1.4)	1	ref	0.0027
High	542	26 (4.8)	2.5	1.4-4.4	
Contact with livestock					
No	724	7 (1.0)	1	ref	0.0006
Yes	3009	63 (2.1)	3.7	1.6-8.6	
Contact with foetal secretions					
No	2159	27 (1.3)	1	ref	0.0169
Yes	1574	43 (2.7)	1.9	1.1-3.1	
Consumption of raw milk					
No	2848	49(1.7)	1	ref	0.1886
Yes	885	21(2.4)	1.5	0.8-2.5	
Consumption of cheese					
No	280	8 (2.9)	1	ref	0.8277
Yes	3453	62 (1.8)	1.1	0.5-2.4	
Consumption of placenta and foetus					
No	3528	60(1.7)	1	ref	0.0169
Yes	205	10(4.9)	2.7	1.3-5.5	
Consumption of blood					
No	3424	58(1.7)	1	ref	0.0515
Yes	309	12(3.9)	2.0	1.0-4.0	
Province**					
Carchi	649	16 (2.5)			
Esmeralda	195	5 (2.6)			
Imbabura	1032	13 (1.3)			
Manabí	377	11 (2.9)			
Pichincha	1480	25 (1.7)			

702

703

Legend: ref, stands for reference category; C.I., confidence interval, *see definition in materials and methods section; ** this variable was used as a random effect in the logistic regression analysis. The p-values are based on the likelihood ratio test comparing the random intercepts-only model and the random effects model with each covariate in turn.

710 **Table IV.** Final model of risk factors associated with human brucellosis sero-positivity
711 among people in the north-western part of Ecuador based on a multivariate random
712 effects logistic regression analysis
713

Risk factors	**OR**	**P-value**	**95% Confidence Interval**
Consumption of foetus and placenta			
No	1	-	ref.
Yes	2.5	0.016	(1.18-5.22)
Contact with livestock			
No	1	-	ref.
Yes	3.0	0.014	(1.25-7.08)
Occupational hazard group			
Low	1	-	ref.
High	1.8	0.049	(1.00-3.35)
Variance components	**Estimate**	**S. E.**	
Canton	**115**	**081**	**(072-185)**
Province	**00**	**029**	*****

714
715 Legend: *, 95% Confidence interval was not estimated by the model; ref, stands for
716 reference category.
717
718

719 **Table V.** Results of blood cultures from patients with high serological titres concomitant with brucellosis presumptive clinical symptoms (North-
720 West Ecuador)

Nº	Province	Age (year)	Sex	Occupation	Contact with livestock	Contact with foetal secretions	Consumption of milk	Consumption of placenta and foetus	Consumption of blood	RB	SAT-EDTA (UI/ml)	iELISA (U/ml)	Blood culture
1	Pichincha	28	M	Farmer	+	+	boiled	-	-	+	-	60	-
2	Pichincha	22	M	Farmer	+	+	boiled	-	-	+	100	60	-
3	Pichincha	17	M	Student	+	-	boiled	-	-	+	80	60	-
4	Pichincha	49	F	Farmer	+	+	raw	-	-	-	80	-	-
5	Pichincha	28	M	Farmer	+	+	boiled	-	-	-	-	30	-
6	Pichincha	49	F	Farmer	+	-	raw	-	-	+	100	60	-
7	Pichincha	50	M	Farmer	+	+	boiled	-	-	-	50	-	-
8	Pichincha	39	M	Farmer	+	+	boiled	+	-	+	100	60	-
9	Pichincha	41	M	Veterinary lecturer	+	+	boiled	-	-	+	80	60	-
10	Pichincha	26	M	Farmer	+	+	raw	+	-	+	100	60	-
11	Pichincha	39	M	Farmer	+	+	boiled	-	-	+	100	60	-
12	Pichincha	41	M	Farmer	+	+	boiled	+	+	-	100	60	-
13	Pichincha	39	F	Slaughterhouse worker	+	+	boiled	-	-	+	50	60	-
14	Carchi	42	M	Slaughterhouse worker	+	+	boiled	-	-	+	40	60	-
15	Carchi	55	M	Transporter	+	-	raw	-	-	+	-	50	-
16	Carchi	50	F	Slaughterhouse worker	+	+	raw	-	-	+	-	48.6	-
17	Carchi	66	F	Slaughterhouse worker	+	+	raw	+	+	+	60	60	-
18	Carchi	36	F	Slaughterhouse worker	+	+	boiled	-	-	+	320	14.4	+
19	Carchi	35	M	Slaughterhouse worker	+	+	boiled	-	-	+	100	26.2	-
20	Pichincha	27	M	Veterinary student	+	-	boiled	-	-	+	1600	60	+
21	Carchi	58	M	Farmer	+	-	raw	-	+	+	800	60	+
22	Carchi	21	M	Farmer	+	+	raw	-	-	+	960	60	-

721
722
723 Legend: RB: Rose Bengal; SAT-EDTA: Wright's Slow Agglutination Test; iELISA: indirect Enzyme Linked Immunosorbent Assay.

Table VI. *In vitro* characteristics of the isolations (group with people under high risk)

					Growth on colorants				Agglutination with serum	
Sample code serology	**Sample code bacteriology***	**Urease activity**	**CO_2 for growth**	**H_2S production**	**Thionine 20 µg**	**Thionine 10 µg**	**Basic fuchsin 20 µg**	**Safranin 100 µg**	**A**	**M**
SHB-Cam-Nor-152	Ec-CIZ-Hum1	+	+	+	-	-	+	+	-	+
SHB-Ay-10	Ec-CIZ-Hum2	+	+	+	-	-	-	-	-	+
SHB-Zon-Nor-370	Ec-CIZ-Hum3	+	+	+	+	-	+	+	-	+

Legend: *= blood culture.

731 **Table VII.** Human brucellosis: symptoms and frequency (N = 70 inhabitants sero-

732 positive to at least one of the three diagnostic tests for brucellosis)

733

Symptoms	Positive cases	%
Muscular pain	29	41.43
Joint pain	25	35.71
Fever	17	24.29
Debility	17	24.29
Headache	16	22.86
Nocturnal sweating	13	18.57
Cardiac problems	10	14.29
Anorexia	4	5.71
Insomnia	4	5.71
No apparent symptoms	17	24.29

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