

Are brucellosis, Q fever and melioidosis potential causes of febrile illness in Madagascar?



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ABSTRACT

Brucellosis, Q fever and melioidosis are zoonoses, which can lead to pyrexia. These diseases are often under-ascertained and underreported because of their unspecific clinical signs and symptoms, insufficient awareness by physicians and public health officers and limited diagnostic capabilities, especially in low-resource countries. Therefore, the presence of *Brucella* spp., *Coxiella burnetii* and *Burkholderia pseudomallei* was investigated in Malagasy patients exhibiting febrile illness. In addition, we analyzed zebu cattle and their ticks as potential reservoirs for *Brucella* and *C. burnetii*, respectively. Specific quantitative real-time PCR assays (qPCRs) were performed on 1020 blood samples drawn from febrile patients. In total, 15 samples (1.5%) were *Brucella*-positive, mainly originating from patients without travel history, while DNA from *C. burnetii* and *Bu. pseudomallei* was not detected.

Anti-*C. burnetii* antibodies were found in four out of 201 zebu serum samples (2%), whereas anti-*Brucella* antibodies could not be detected. *Brucella* DNA was detected in a single zebu sample. Three out of 330 ticks analyzed (1%) were positively tested for *C. burnetii* DNA but with high Ct values in the qPCR assay. Our data suggest that zebus as well as *Amblyomma* and *Boophilus* ticks have to be considered as a natural reservoir or vector for *C. burnetii*, but the risk of cattle-to-human transmission is low. Since bovine brucellosis does not seem to contribute to human infections in Madagascar, other transmission routes have to be assumed.

1. Introduction

Brucella spp., *Coxiella burnetii* and *Burkholderia pseudomallei* are Gram-negative pathogens, which cause brucellosis, Q fever, and melioidosis, respectively. They may considerably affect human and

animal health, especially in low-resource areas. While Q fever and brucellosis are classical zoonotic diseases, melioidosis is a saprozoosis, indicating that the source of infection is the abiotic environment (e.g. contaminated soil or water) (Hubalek, 2003). In areas endemic for malaria and typhoid fever, zoonotic infections, often characterised by

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non-specific clinical symptoms such as fever, headache, and fatigue, are underdiagnosed (Crump et al., 2013; Halliday et al., 2015). Hence, physicians should be aware of zoonotic diseases as a cause of fever of unknown origin (FUO) (Cleri et al., 2007).

Worldwide, more than 500,000 human brucellosis cases are reported annually, with high incidences in the Middle East and Central Asia (e.g. Syria, Iran, Iraq, Mongolia, Kirgizstan) and North Africa (e.g. Algeria) (Pappas et al., 2006). Human disease is mainly caused by *Br. melitensis*, *Br. abortus* and *Br. suis* which are transmitted to humans through direct contact with infected livestock (i.e. sheep, goat, cattle, pig), their excretions (faeces, urine, placenta and abortions) or by ingestion of contaminated food products (mainly unpasteurized dairy products and raw meat). In livestock farming, brucellosis leads to tremendous economic losses due to decreased milk production, abortions and limited fertility (McDermott and Arimi, 2002).

With the exception of New Zealand and French Polynesia, Q fever has a worldwide distribution (Million and Raoult, 2015). The largest Q fever outbreak so far has been notified in the Netherlands with more than 4000 human cases between 2007 and 2010 (Schneeberger et al., 2014). According to a recent review covering 51 studies, Q fever seroprevalence in the general African population is less than 8% with slightly higher rates in children (10–17%). However, it varies widely among geographic regions and a much higher seroprevalence was found in Egypt (32%) (Vanderburg et al., 2014). The main reservoirs of *C. burnetii* are mammals and arthropods, with ruminants (sheep, goat, cattle) as the most common source of human infections. The disease is usually transmitted through inhalation of aerosols from amniotic fluid or placenta material of infected animals, although ingestion, transfusion and sexual intercourse may sporadically occur (Million and Raoult, 2015). Acute, chronic (endocarditis) or subclinical courses have been described after infection with *C. burnetii*. In livestock, Q fever can lead to a loss of productivity mostly due to increased abortion rates (Vanderburg et al., 2014).

Melioidosis is a tropical disease, which is predominantly reported in Southeast Asia (i.e. Thailand, Singapore, Vietnam, Malaysia) and North Australia, although sporadic cases also occur elsewhere. In a recent study on the global burden of melioidosis, a total of 165,000 cases including 89,000 fatalities per year were estimated worldwide. These estimates were associated with substantial uncertainty (Limmathurotsakul et al., 2016). The reported number of cases in tropical low-resource countries are likely to be underestimated due to insufficient disease awareness and absence of diagnostic facilities (Hoffmaster et al., 2015). The host species spectrum of *Bu. pseudomallei* is broad including pigs, sheep, goats, horses, dogs and cats. A wide range of clinical symptoms have been described in humans, e.g. fever, headaches, muscle pain, pneumonia with chest pain and cough, abscesses. Humans are infected through contact with contaminated surface water and mud or indirectly through the inhalation of contaminated aerosols (Heymann, 2008).

Madagascar is a large island situated in the Indian Ocean, 500 km east of Mozambique, with an area of 587,295 km². The island is divided into 22 regions with a total population of 23,400,000 (INSTAT, 2016). Its climate is tropical along the coast, temperate in the Central Highlands and arid in the South. Livestock is mainly composed of cattle (ca. 10 million), goats (ca. 1,470,000), sheep (ca. 840,000) and pigs (ca. 1,500,000) (Data 2012: FAOSTAT (2015)). Recent epidemiological data on the presence of brucellosis (McDermott and Arimi, 2002) and Q fever (Vanderburg et al., 2014) in Madagascar do not exist and only sporadic cases of melioidosis have been reported (Garin et al., 2014).

Therefore, our objective was to investigate the presence of *C. burnetii*, *Brucella* and *Bu. pseudomallei* in Malagasy patients suffering from febrile illness as a potential sentinel symptom for zoonotic diseases. Furthermore, we aimed to identify potential sources of human infections, by screening zebus for *Brucella* and both zebus and ticks for *C. burnetii*.

2. Materials and methods

2.1. Human study population and survey area

The International Vaccine Institute (IVI) collected blood samples within the Typhoid Fever Surveillance in Africa Program (TSAP). This network aims to generate data on the burden of typhoid fever and other invasive *Salmonella* infections through standardized surveillance and disease burden studies in ten African countries (von Kalckreuth et al., 2016).

The TSAP survey sites in Madagascar consisted of public health care facilities in central Madagascar, both in a rural environment (Imerintsiasosika) and urban slums (Isotry, located in the capital of Madagascar Antananarivo) with a catchment population of 46,000 and 70,000 inhabitants, respectively. In the rural catchment area, cattle husbandry and rice farming are predominant and the people live in close contact with their animals. In total, 18% and 9% of the population in Imerintsiasosika and Isotry sought care for fever in the surveillance facilities (Panzner et al., 2016). A total of 4500 whole blood EDTA samples were collected in Madagascar between 2011 and 2013 within the framework of TSAP from patients with unknown diagnosis at presentation and a body temperature ≥ 37.5 °C. From these, a subset of 1020 samples taken from patients with pyrexia ≥ 38.5 °C was screened for *C. burnetii*, *Brucella* spp. and *Bu. pseudomallei*. The selection of the subset was motivated in anticipation of a greater chance to detect bacteraemia in patients with a higher body temperature. However, we cannot exclude that in the samples not analyzed (≥ 37.5 °C and < 38.5 °C) we might have missed subclinical or chronic infections. Samples were stored at -20 °C prior to testing. Demographic data (age, gender), place of residence, travel history, clinical signs and symptoms as well as medication use were recorded.

Informed written consent was obtained from the patients, their parents or their legal guardians. The study was approved by the Malagasy Ethical Committee (no. 045 MSANP/CE) and the IVI Institutional Review Board (no. IVI IRB #2011-001).

2.2. Sampling of zebu and ticks

In October 2012, a total of 215 zebu cattle were sampled in three slaughterhouses in the municipality of Bemasoandro (district of Antananarivo-Atsimondrano). Origin of the cattle, age, sex, health and nutritional status were recorded.

Both whole blood and serum samples were taken, 214 samples could be used for bacteriology and qPCR, and 201 samples for serology.

In total, 1822 ticks (1090 *Amblyomma variegatum* and 732 *Rhipicephalus (Boophilus) microplus*) were collected with a maximum of 10 ticks sampled from each cattle as described previously by Keller et al. (2016). A random sample of 330 ticks (including 199 *Amblyomma variegatum* and 131 *Rhipicephalus microplus* ticks) from 80 cattle was used for screening.

2.3. Molecular and serological tests

2.3.1. DNA preparation from blood samples and ticks

Genomic DNA was prepared from 1 ml of human EDTA blood samples using the FlexiGene DNA Kit (Qiagen, Hilden, Germany). For zebu blood samples either FlexiGene DNA Kits (Qiagen) or High Pure Template Preparation Kits (Roche Diagnostics GmbH, Mannheim, Germany) were used. After mechanical disruption DNA was extracted from ticks by the QIAamp DNA Mini Kit (Qiagen) as previously described (Keller et al., 2016).

2.3.2. *Brucella* spp. detection

Quantitative real-time PCR assays (qPCR) were performed to detect brucellae in human and zebu samples by targeting the genus specific marker sequences *bcsp31* and IS711 (Al Dahouk et al., 2007; Cloeckaert

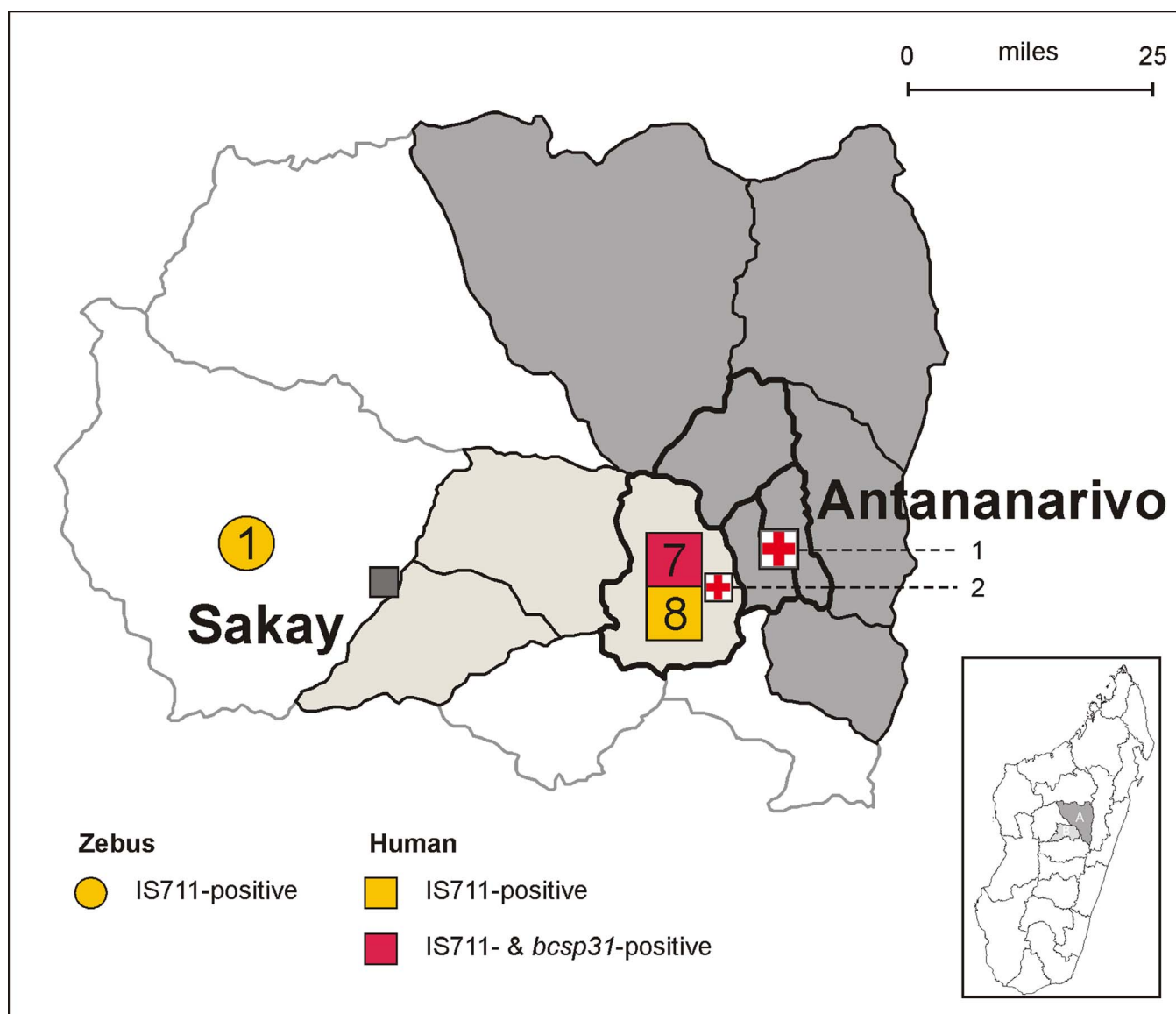


Fig. 1. Brucellosis cases in febrile patients originating from the regions Analamanga (A) and Itasy (B), Madagascar, 2011–2013. Diagnosis was based on the detection of *Brucella*-specific target sequences in human blood samples, both IS711 and *bcsp31* ($n = 7$, red squares) or solely IS711 ($n = 8$, yellow square). A single IS711-positive zebu (yellow circle) was detected in the Tsiroanomadidy district (Bongolava region) in 2012. The Red Cross represents the sampling sites in Antananarivo (basic health centre CSB II Isotry and clinic CHU Tsaralalana) and in Itasy (basic health centre CSB II Imerintsiasika).

et al., 2000). The IS711 qPCR is supposed to be specific and highly sensitive for the detection of the genus *Brucella*, with an analytical sensitivity of 2 fg (Bounaadja et al., 2009). Specificity and sensitivity of the *bcsp31* qPCR are also known to be high, but its limit of detection ranges between 16 and 18 fg (Al Dahouk et al., 2007). For qPCR, the Quantifast Pathogen Detection Kit (Qiagen, Hilden, Germany) with an internal amplification control was used. Each DNA sample was analyzed in duplicate against positive and negative matrix controls according to the MIQE guidelines (Bustin et al., 2010). A sample was considered *Brucella*-positive if the genus specific IS711 qPCR showed a positive test result. The result was confirmed by the qPCR targeting *bcsp31*. A threshold cycle value (Ct) > 40 was deemed to be false positive and considered negative.

Zebu serum samples were screened for anti-*Brucella* antibodies using ELISA (IDEXX Brucellosis Serum X2 Ab Test; IDEXX, Hoofddorp, the Netherlands), serum agglutination test and complement fixation test (Pourquier, IDEXX, Hoofddorp, the Netherlands) according to manufacturers' instructions. Furthermore, whole blood samples were transferred to a selective biphasic culture medium (Brodie and Sinton, 1975;

Corner et al., 1985; Ruiz Castaneda, 1961), incubated at 37 °C with and without 10% CO₂ (Alton et al., 1975) and potential growth of bacteria was monitored weekly on the solid phase of the culture flasks. Growing bacteria were identified by MALDI-TOF MS (Karger et al., 2013). If no colonies were observed, the flasks were shaken and further incubated. In the absence of growth, samples were finally declared negative after six weeks.

2.3.3. *Coxiella burnetii* and *Bu. pseudomallei* detection

To detect *C. burnetii* in human and tick samples, specific and highly sensitive qPCRs targeting IS1111 were used (Klee et al., 2006). For the detection of *Bu. pseudomallei* in human samples, we carried out qPCRs targeting *flaC* (Tomaso et al., 2005; Tomaso et al., 2004).

We also tested for *C. burnetii* Phase I/II IgG antibodies in zebu serum samples by a commercially available ELISA (IDEXX Q Fever Ab Test; IDEXX Switzerland AG, Liebefeld-Bern, Switzerland) according to the manufacturer's guidelines (with S/P defined as the ratio between optical density (OD) of the sample (S) and OD of the positive control (P), S/P ≥ 40% was considered positive, results in the interval

30% \leq S/P < 40% were considered to be doubtful and S/P < 30% was interpreted as negative). The values of test accuracy provided by the manufacturer (*personal communication*) were 0.93 for sensitivity and 1.0 for specificity. However, the test has never been validated in zebu.

2.4. Statistical analysis

The proportion of positive samples and respective 95% confidence intervals (95% CI) were determined assuming a binomial distribution. Fisher's exact test and Wilcoxon rank sum test were used to compare categorical data and continuous non-normal data, respectively. We calculated odds ratios (OR) with 95% CIs to describe the strength of associations between the infection status and independent variables. P-values < 0.05 were considered significant. Statistical analysis was performed using STATA 13.1 (StataCorp, TX, USA).

3. Results

3.1. Characteristics of the study population

Patients presented to the children's ward (n = 29; 3%), inpatient (n = 3) or outpatient departments (n = 988; 97%) of two basic health centres (CSB II Isotry, Antananarivo, Analamanga region (n = 627; 61%); CSB II Imerintsiasika, Itasy region (n = 389; 38%)) and one clinic (CHU Tsaralana, Antananarivo (n = 4)) from September 2011 to May 2013. The district of residence was known for 1011 out of 1020 febrile patients. In total, 626 (61.4%) patients originated from the central region of Analamanga (620 patients from the urban district of Antananarivo-Renivohitra, the country's capital, and six patients from the bordering districts Atsimondrano, Ambohidratrimo and Avaradrano). A total of 385 (37.7%) patients were residents of the Arivonimamo district in the central region of Itasy. The median age of the patients (n = 1020) was 21 years (range 0–79, IQR 11; 34), and 611 (60%) were females. Their body temperature ranged from 38.5 to 41 °C (median 39 °C, IQR 38.6; 39.2). The date of onset of symptomatic febrile illness is shown in Fig. 2. The patients mainly suffered from clinical symptoms of flu-like or gastrointestinal disease (Table 1). Most of them were treated symptomatically with nonsteroidal anti-inflammatory drugs and less than 20% received causal treatment on spec (i.e. antibiotics or antimalarial medication).

3.2. Prevalence of *Brucella*, *C. burnetii*, *Bu. pseudomallei* in febrile patients

The *Brucella*-specific molecular marker IS711 was detected in 15 samples (1.5%, 95% CI [0.8–2.4]). Of these, seven samples were confirmed by the detection of *bcs*p31. The *Brucella*-positive samples

were obtained from eight females and seven males, aged 7–65 years (median 24), who were recruited in the outpatient department of the CSB II Imerintsiasika. All cases were residents of rural villages in the district of Arivonimamo (Itasy region) (Fig. 1, Table 1). Thirteen cases felt ill from 21st September through 26th December 2011, and two cases from 28th August through 3rd September 2012 (Fig. 2). There were no significant associations between human brucellosis and age (p = 0.25; Wilcoxon rank sum test) or gender (p = 0.60; Fisher's exact test) of the cases (Table 1). Brucellosis cases presented less frequently with a cough (13% vs. 55%; OR 0.14, 95% CI [0.01–0.57]) or headaches (53% vs. 81%; OR 0.26, 95% CI [0.08–0.86]) than other febrile patients. In contrast, brucellosis cases were more likely to suffer from arthralgia (27% vs. 3%; OR 12.7, 95% CI [2.8–46.0]) than non-brucellosis febrile patients (Table 1).

Only a single case travelled outside of her local residence 7 days before the onset of the current illness.

Neither *C. burnetii* nor *Bu. pseudomallei* DNA was detected in the investigated human blood samples.

3.3. Prevalence of *Brucella* and *C. burnetii* in zebu and ticks

All zebu were male (213/215, while two zebu lacked information on sex), aged between 4 and 20 years (median: 8 years) and three animals were clinically identified to be sick. The zebu originated from the regions Bongolava (n = 83), Haute Matsiatra (n = 66), Menabe (n = 31), Sofia (n = 23), Vakinankaratra (n = 11) and Itasy (n = 1). *Brucella* DNA (IS711-marker) was detected in a single eight years old healthy zebu (0.5%, 95% CI [0.01–2.6]) originating from the Bongolava region. Brucellosis could not be serologically or microbiologically verified.

Anti-*C. burnetii* antibodies were detected in four out of 201 available serum samples of adequate quality (2%, 95% CI [0.5–5.0]), all originating from zebu aged seven to eight years old, of the Bongolava region (Fig. 3). In addition, six samples suspected to be positive, originated from Vakinankaratra (2), Menabe (2), Haute Matsiatra (1) and Bongolava (1) (Fig. 3).

Three out of 330 ticks analyzed (1%), including two *Amblyomma variegatum* and one *Rhipicephalus microplus*, were positively tested for *C. burnetii* DNA but with high Ct values (39.1, 39.9, 41.4) in the qPCR assays.

4. Discussion

To the best of our knowledge, this survey represents the first evidence of human brucellosis in febrile patients in Madagascar. Indeed, a single seropositive case had been reported in 1982, but the

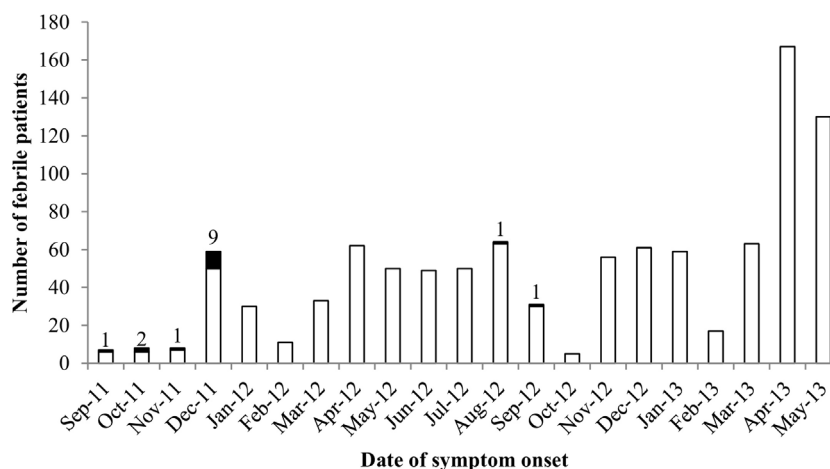


Fig. 2. Date of onset of symptomatic disease in febrile patients, Madagascar, September 2011–May 2013 (n = 1006; 14 patients without symptom onset information are not represented). *Brucella* IS711 positive patients are marked in black (n = 15).

Table 1Demographics and clinical features of febrile patients with (n = 15) and without (n = 1005) *Brucella* infection, defined by molecular detection of IS711, Madagascar, 2011–2013.

	Brucellosis cases (N = 15)	Febrile patients without brucellosis (N = 1005)		
	n/N (%)	n/N (%)	P value	OR [95% CI]
Demographics				
Male sex	7/15 (46.7)	402/1005 (40.0)	0.61	1.31 [0.40–4.18]
Age (years): median (IQR)	24 (14; 35)	21 (11; 34)	0.25	
Rural	15/15 (100)	370/996 (37.2)	< 0.001*	Undefined
Clinical symptoms and signs				
Median body temperature (°C) on admission (IQR)	39 (38.5; 39.7)	38.9 (38.6; 39.2)	0.46	
Diarrhea	4/15 (26.7)	221/1005 (22.0)	0.75	1.29 [0.30–4.41]
Headache	8/15 (53.3)	817/1005 (81.3)	0.01*	0.26 [0.08–0.86]
Constipation	0/15 (0)	23/1005 (2.29)	0.55	0 [0.00–11.20]
Sore throat	3/15 (20)	353/1005 (35.1)	0.28	0.46 [0.08–1.73]
Rash	0/15 (0)	14/1005 (1.39)	1.00	Undefined
Cough	2/15 (13.3)	549/1005 (54.6)	0.003*	0.14 [0.01–0.57]
Vomiting	3/15 (20)	231/1005 (23)	1.00	0.84 [0.15–3.14]
Arthralgia	4/15 (26.7)	28/1005 (2.8)	< 0.001*	12.69 [2.75–45.99]
Hospital admission	0/15 (0)	6/995 (0.6)	1.00	Undefined
Use of medication prior to consultation				
Analgesics	7/15 (46.7)	393/836 (47.0)	1.00	0.99 [0.30–3.14]
Antibiotics	2/15 (13.3)	171/770 (22.2)	0.54	0.54 [0.06–2.42]
Antimalaria	0/13 (0)	19/675 (2.8)	1.00	Undefined

OR: odds ratio, CI: confidence interval, IQR: interquartile range.

* P < 0.05.

source of infection could not be clarified and whether it was an autochthonous case remained doubtful (Ribot and Coulanges, 1982).

All brucellosis cases identified in our study lived in rural villages of the district Arivonimamo, suggesting an endemic cluster or an outbreak. In contrast, no cases were detected among patients resident in the country's capital. Despite the considerable sample size, the catchment area only covered central regions of Madagascar and may therefore not be representative for the whole country.

As the incubation period may extend to several months (Heymann, 2008), travel-associated infections cannot be ruled out but only one case had a recent travel history. In 13 cases, the reported disease onset spanned three months (September–December 2011), while in two cases the symptoms occurred within one week (end of August–beginning of September 2012). Since data for backtracking are missing, we do not know whether these cases were epidemiologically linked, whether they were in contact with infected animals or associated with abortions in ruminants or whether they had consumed contaminated animal products (non-pasteurized dairy products or undercooked/raw meat).

In our study, brucellosis cases were more likely to suffer from arthralgia than febrile patients without brucellosis. Although clinical symptoms of human brucellosis are not pathognomonic, arthralgia is frequently described (in 65% of the cases, 95% CI [58–72]), according to a meta-analysis by Dean et al. (2012). In contrast, the number of cases suffering from arthralgia was quite low in the present study (27%), but in line with studies from India (24%) and Kuwait (21%) (Mantur et al., 2006; Mousa et al., 1988). The phenomenon that coughing is less likely among cases compared to other febrile patients was already observed in a study on human brucellosis in Tanzania (Bouley et al., 2012). As *Brucella* DNA can persist for years after clinical cure and in the absence of symptoms indicative of disease persistence or relapse, our study population may have comprised acute, chronic or even asymptomatic brucellosis cases with fever of hitherto unknown origin (Vrioni et al., 2008). Eight IS711-positive samples could not be confirmed using the *bcs31* qPCR which can be explained by the high copy number of IS711 resulting in a higher sensitivity, whereas *bcs31* only exists in a single copy in the *Brucella* genome.

All zebu samples were seronegative and except for a single case (IS711 positive, but *bcs31* negative) *Brucella* DNA could not be found. The detection of *Brucella* in only one animal among 201 seronegative zebus from various flocks might be explained by a spill-over of a non-

host specific *Brucella* species, such as *Br. melitensis* from sheep or goats, and a low infection pressure or a spill-over from wildlife. So far, only *Br. suis* biovar 5 has been isolated, from a bitch in 1973 (Verger et al., 1975), and there is no further evidence of the occurrence of brucellosis on the island.

Malagasy zebu, which compose 80% of the cattle population (Meyer, 2016), are related to East African shorthorn zebu on the African mainland (Decker et al., 2014). According to official data from FAOSTAT (2015), livestock animals are rarely imported. Since the 1990s a total of 2870 heads of cattle have been imported from New Zealand (in 2005/2006), which is officially free from bovine brucellosis, and 52 sheep and nine pigs have been imported from France (FAOSTAT, 2015). In addition, four goats were imported from Comoros in 2007, followed by 208,300 goats imported from Australia in 2008. However, the real volume of livestock trade in Madagascar might be underestimated in the FAO database.

Brucellosis is highly prevalent in neighbouring Tanzania (3.5–10% seroprevalence in humans, 21.5–48% seroprevalence in dairy cattle) (Bouley et al., 2012; Mathew et al., 2015; Shirima et al., 2014) and in Mozambique, where a seroprevalence of 27% was detected in buffaloes (Tanner et al., 2015). In contrast, on the nearby island of Mauritius, *Br. abortus* was isolated just once from a foetus aborted by a cow in 1947 and human brucellosis was only suspected to occur (Webb and Webb, 1948). According to our data, zebu do not seem to play a role in the transmission of *Brucella* spp. in Madagascar, which suggests that other reservoir species may be involved (i.e. small ruminants, pigs, wildlife or pets). In Madagascar, cattle farming is mostly concentrated in the north-western, the western coast and the southern part of the island, whereas 85% of goat and sheep farms are located in the South, and pig rearing is mainly found in central Madagascar (MAEP, 2007). Due to the rather isolated geographical location of Madagascar, we hypothesize humans could have been infected by other potentially endemic *Brucella* spp. distinct from *Br. abortus*.

Although human samples were not found to be *C. burnetii*-positive, a seropositivity of 2% was detected in zebus, which suggests a low risk for the Malagasy population to acquire Q fever. False positive serological test results cannot be excluded but *C. burnetii* DNA was detected in ticks, indicating that the bacterium is actually present on the island. In Madagascar, only a few human Q fever cases were reported in the fifties and sixties (Courdurier et al., 1952; Guiot and Lemaigre, 1964). The

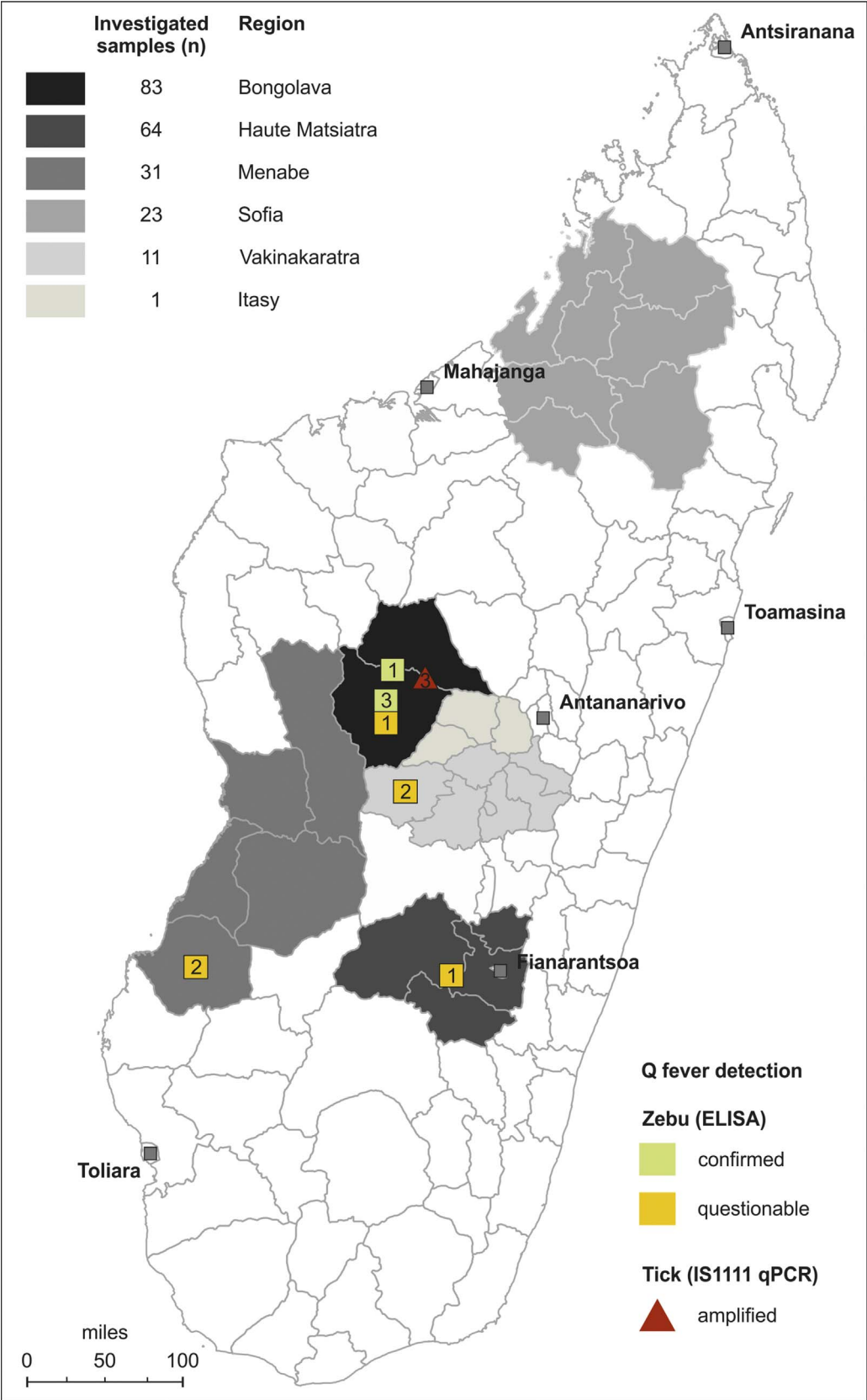


Fig. 3. Regions of origin of the sampled zebu, Madagascar, 2012 (in different grey scales). *C. burnetii* seropositive zebu cattle (green squares, n = 4), suspected positive zebu cases (yellow squares, n = 6), qPCR-positive ticks (red triangle).

first clinical case confirmed by serology was described in 1959 (Porte et al., 1959). In 1964, a Q fever outbreak affected 30 patients suffering from a typhoid-like syndrome and atypical pneumonia (Guiot and Lemaigre, 1964). On neighbouring islands of Madagascar, *C. burnetii* was detected in humans (Artagnan et al., 2008) and ruminants in Reunion (Cardinale et al., 2014). In 1992, a seroprevalence of 5.4% was reported in healthy blood donors from the Comoros (Dupont et al., 1995) and in 2012, a case of Q fever was diagnosed in a patient returning from the same archipelago (Brouqui et al., 2005). The seroprevalence in cattle in Madagascar (2%) was lower than in neighbouring Reunion (11.8%) (Cardinale et al., 2014) and on mainland Africa, where the *C. burnetii* seroprevalence ranged between 4% in Senegal and 32% in Cameroon (Vanderburg et al., 2014). However, seroprevalence comparisons should be interpreted with caution due to differences in the sampling design, sample size and diagnostic tests used. In the present study, only male zebu were sampled which may not be the population with the highest *C. burnetii* seroprevalence in Madagascar. In Denmark for instance, a significantly higher seroprevalence was observed among dairy cattle compared to beef breeds, and in females compared to males (Paul et al., 2014).

No evidence for *Bu. pseudomallei* was found among the febrile patients investigated. A recent study predicted an annual melioidosis incidence of 880 cases (95% credible interval [326–2464]) per 100,000 population in Madagascar suggesting that the disease is considerably underreported. There are large differences in environmental suitability for *Bu. pseudomallei* persistence on the island (Limmathurotsakul et al., 2016). Central Madagascar where our sampling sites were located, revealed a lower predicted environmental suitability for *Bu. pseudomallei*, which might explain the absence of clinical cases in the catchment area. In 1936, *Bu. pseudomallei* had already been isolated from a pig in Madagascar (Girard, 1936) and later also from soil samples collected on the island (Galimand and Dodin, 1982). Autochthonous human cases of melioidosis have recently been reported (Garin et al., 2014) as well as cases in travellers returning from Madagascar (Borgherini et al., 2006; Ezzedine et al., 2007). From time to time sporadic cases are notified in the Indian Ocean region (Borgherini et al., 2015; Issack et al., 2005).

As the qPCR assays used did not allow for *Brucella* spp. differentiation and the role of reservoir animals (other than zebu) could not be assessed, further studies both in humans (especially in high risk groups such as farmers, shepherds, butchers, food factory workers, veterinarians) and in animals (including other ruminants and wildlife) are required in Madagascar, and should be complemented by *Brucella* species identification and genotyping to establish possible geographic and evolutionary associations. To fully appraise the burden of brucellosis and Q fever in Madagascar, future sampling strategies should include regions where livestock production (cattle, sheep/goat, and pig) is concentrated, as well as wildlife. The current study suggests an underdiagnosis of brucellosis cases in Madagascar, which may be explained by a lack of disease awareness due to unspecific clinical signs and symptoms. Hence, specialised laboratory facilities have to be established for future surveillance. It should be evaluated whether syndromic surveillance of abortions among ruminants can contribute to the early identification of *Brucella* spp. of public health importance and possible Q fever cases. With respect to *Bu. pseudomallei*, environmental samples (e.g. from rice paddy fields) are currently investigated.

In summary, based on the current study and literature data, there is sufficient evidence for the occurrence of *Brucella* spp., *C. burnetii* and *Bu. pseudomallei* on the island of Madagascar. Whether the virulence of the pathogens, the genetic background of animal hosts or climate conditions contribute to the low number of human cases remains to be investigated. Nevertheless, brucellosis, Q fever and melioidosis have to be taken into account in the differential diagnosis of febrile illnesses both in Malagasy patients and in travellers returning from Madagascar.

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