Detection of *Brucella abortus* in Chiredzi district in Zimbabwe

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Brucellosis is an endemic disease in Zimbabwe caused by the genus *Brucella*. *Brucella* seroprevalence was recently reported to be high in the wildlife-livestock interface in the Chiredzi district and the neighbouring Gonarezhou National Park (GNP) in Zimbabwe, and higher amongst communal cattle with an abortion history and access to grazing in GNP than amongst communal cattle with no abortion history or access to grazing in GNP. The aim of this study was to investigate *Brucella* species in brucellosis seropositive cattle in the Chiredzi district with access to GNP using isolation and identification. Isolation of *Brucella* species from whole blood (n = 18) and milk samples (n = 10) from seropositive animals with an abortion history was based on the rose Bengal test (RBT) and enzyme-linked immunoassays (enzyme-linked immunosorbent assay [ELISA]; indirect ELISA and complement ELISA), using microbiology and polymerase chain reaction (PCR) methods. *Brucella abortus* was cultured and identified from blood and milk collected from seropositive cows in both communal areas. The *Brucella*-specific 16-23S intergenic spacer (ITS) PCR and multiplex AMOS-PCR assays verified the identification of the cultures. Our results confirmed that *B. abortus* is present in cattle on communal farms in the Chiredzi district in Zimbabwe and might cause cattle abortions. The need for implementing control measures and raising public awareness on zoonotic transmission of brucellosis are recommended.

Introduction

Bovine brucellosis is a bacterial disease caused by *Brucella abortus*. In addition to its zoonotic importance, it also affects animal health and production (Godfroid *et al.* 2005; Pappas *et al.* 2005). Consumption of contaminated foods or occupational exposure remains the major source of infection in humans. Brucellosis is primarily recognised as an occupational hazard for veterinarians, farmers, laboratory technicians, slaughterhouse workers, and others who work with animals and their products. The main source of infection for the public is through ingestion of unpasteurised dairy products. The bacteria can also be transmitted through raw or undercooked meat from infected animals. The *Brucella* species generally considered pathogenic for humans, in decreasing order of virulence, are *Brucella melitensis*, *Brucella suis* and *B. abortus* (Baldwin & Goenka 2006).

Identification of *Brucella* spp. is important in surveillance and eradication efforts. Currently, mainly serological screening of potential hosts and to a lesser extent isolation and identification of the pathogen from potential hosts are used for the diagnosis of brucellosis. Culturing (isolation and identification) of *Brucella* spp. is recognised as the 'gold standard', but is time consuming and complex, and positive animals sometimes yield negative culture results (Alton *et al.* 1988; OIE 2008; Whatmore 2009). Most *Brucella* cultures have been isolated from aborted foetuses, milk, hygroma fluid, or lymph nodes from infected animals. Madsen (1989) and Mohan *et al.* (1996) identified *B. abortus* biovar 1 cultures from aborted foetuses. The same biovar (bv) was also isolated from an aborted foetus of a waterbuck in Wankie (Hwange) National Park (Condy & Vickers 1969) and from eland hygroma fluid on a game ranch in Zimbabwe (Condy & Vickers 1972). Matope *et al.* (2009) isolated primarily *B. abortus* bv 1 and to a lesser extent *B. abortus* bv 2 from aborted foetuses and milk samples from infected herds in Zimbabwe. These authors also isolated *B. melitensis* bv 1 from an aborted foetus of a goat in Zimbabwe (Matope *et al.* 2009). It is difficult to obtain positive *Brucella* cultures from blood and positive cultures are only obtained from 10% – 70% of infected human infections since successful isolation depends on the duration, the localisation of the infection and the type of *Brucella* species (Al-Attas *et al.* 2000).

Various polymerase chain reaction (PCR) assays are available for differentiating *Brucella* at the genus, species and/or biovar level. Genus-specific PCR assays like 16-23S rRNA intergenic spacer (ITS) region (Keid *et al.* 2007) detect only *Brucella*, whereas multiplex PCR assays differentiate *Brucella* at the species level (Bricker & Halling 1994, 1995; Garcia-Yoldi *et al.* 2006; Halling, Tatum & Bricker 1993). The automated multiplex oligonucleotide synthesizer (AMOS) multiplex PCR

assay distinguishes *B. abortus* (bv 1, 2 and 4), vaccine strains *B. abortus* RB51 and S19, *B. melitensis* (bv 1, 2 and 3), vaccine *B. melitensis* rev1, *Brucella ovis*, and *B. suis* (bv 1) (Bricker & Halling 1994, 1995; Halling *et al.* 1993).

Brucellosis is endemic in sub-Saharan African countries. In Zimbabwe it was first diagnosed from aborted cattle in 1913 (Bevan 1931). Various studies in the country showed a higher *Brucella* infection in commercial than communal areas (Madsen 1989; Matope *et al.* 2010; Swanepoel, Blackburn & Lander 1976). A recent study in the wildlife-livestock interface (Malipati and Pesvi) and non-interface (Chomupani and Pfumare) communal areas in the south-east lowveld of Chiredzi district (Figure 1) showed a significantly higher *Brucella* seroprevalence in cows with an abortion history and in cattle grazing in parks (Gonarezhou National Park [GNP] and Kruger National Park [KNP]) (Gomo *et al.* 2012). Although bovine brucellosis was demonstrated through serology (Gomo *et al.* 2012), no isolation or characterisation of the bacteria was done. Due to the potential health risk to community members, the objective of the present study was to further characterise the brucellosis species from infected herds in the Malipati and Pesvi communal areas in Chiredzi district, which were found seropositive using the rose Bengal test (RBT) and competitive enzyme-linked immunoabsorbent assay (cELISA) in the study by Gomo *et al.* (2012) and RBT and indirect enzyme-linked immunoabsorbent assay (iELISA) in this study. *Brucella*-specific PCR and AMOS-PCR assays were used to confirm the identity of the *Brucella* isolates.

Materials and methods

Study area and sample collection

The study was conducted in the Chiredzi district in the south-east lowveld of Zimbabwe as described earlier by Gomo *et al.* (2012). The Malipati and Pesvi communal areas in the Chiredzi district share boundaries with the GNP in Zimbabwe and the unfenced region of the northern KNP (separated by the Limpopo River), respectively (Figure 1). The two communal areas were selected based on high *Brucella* seroprevalence, reports of abortion and no history of vaccination (Chiredzi Veterinary Services, pers. comm., 2009; Gomo *et al.* 2012). Samples were collected from cattle at the Malipati and Pesvi dip tanks during 2008 and 2009. The Malipati dip tank is located about 1 km from the unfenced GNP and Pesvi dip tank lies adjacent to the unfenced KNP across the Limpopo river (dip tank 3 km from northern boundary of KNP). Whole blood (n = 18) as well as milk (n = 10) samples (Table 1) were collected from herds with an abortion history and that tested seropositive using RBT and cELISA by Gomo *et al.* (2012). The iELISA was done on samples from Malipati and Pesvi communal cattle (700 serum samples of 1038 tested cattle) that were part of the study of Gomo *et al.* (2012) to confirm their seropositive status.

Cultures

Only milk (n = 10) and blood samples (n = 18) collected from animals which had a history of abortion and had tested

FIGURE 1: The location of the two communal areas (Malipati and Pesvi) that were surveyed in the Chiredzi district in Zimbabwe that borders the Gonarezhou National Park and the Kruger National Park.

TABLE 1: Information of rose Bengal test seropositive bovines from which whole blood and milk samples were collected in the Pesvi and Malipati communal regions in Zimbabwe.

Animal number	Other strain number†	Location	Animal age in months	Sex	iELISA‡
2[a]	9	Pesvi	54	M	P
7[a,c]	139	Pesvi	72	F	N
8[b,c]	150	Pesvi	36	F	P
10[a,c]	**43**	**Malipati**	**72**	**F**	**P**
11[a,c]	357	Pesvi	72	F	N
13[a,c]	437	Pesvi	84	F	N
14[b]	456	Pesvi	156	F	P
15[b]	458	Pesvi	120	F	P
16[a,c]	462	Pesvi	72	F	P
17[b,c]	**323**	**Pesvi**	**84**	**F**	**P**
19[b]	487	Pesvi	144	F	P
20[b]	494	Pesvi	168	F	P
21[a,c]	500	Malipati	72	F	P
22[c]	503	Malipati	72	F	P
31[a,c]	564	Malipati	60	F	P
32[a,c]	577	Malipati	48	F	N
34[b]	593	Malipati	144	F	P
39[b]	615	Malipati	48	F	P
42[b]	717	Pesvi	156	F	P
45[b]	726	Malipati	60	F	P
61[b]	806	Malipati	36	F	P
62[b]	813	Malipati	24	F	P
65[b]	820	Malipati	48	M	P
66[b]	853	Malipati	60	M	P
67[b]	854	Malipati	48	F	P
71[a,b,c]	861	Malipati	48	F	P
75[b]	911	Pesvi	84	F	P
76[b]	913	Pesvi	84	F	N

Bold indicates *Brucella* cultures isolated from milk and blood.
M, Male; F, female; iELISA, indirect enzyme-linked immunoabsorbent assay; P, iELISA results from rose Bengal test seropositive samples positive for the iELISA results; N, iELISA results from rose Bengal test seropositive samples negative for the iELISA results.
[a], Indicates milk samples collected from lactating cows.
[b], Indicates whole blood samples.
[c], Indicates animals with previous abortion history.
†, Strain number used by collector.
‡, The bovine brucellosis iELISA kit (Institut Pourquier) was used according to the manufacturer's instructions at the Department of Veterinary Tropical Diseases, University of Pretoria, South Africa, on serum samples.

positive for *Brucella* antibodies using serological tests were cultured for *B. abortus* isolation at the Central Veterinary Laboratory (CVL) in Harare, Zimbabwe (Table 1). Milk samples were centrifuged at 6000 g – 7000 g for 15 min; skim milk was discarded and the cream and sediment were mixed and spread on *Brucella* selective medium and blood agar (BA) (Quinn *et al.* 1994). The inoculated *Brucella* selective media and BA plates were placed in a jar with gas (6% [oxygen] O_2, 10% [carbon dioxide] CO_2 and 84% [nitrogen] N_2) at 37 °C and examined for 10 days. Plates that did not show any growth after 10 days were discarded as negative. Suspected *Brucella* colonies were transferred to BA, on which *Brucella* appeared small (1 mm diameter), round, grey and non-haemolytic. Suspected colonies were Gram and modified Ziehl Neelsen (Stamp's) stained and the reactions to oxidase and catalase were observed (Quinn *et al.* 1994). Speciation of *Brucella* colonies was done using microbiology tests (excluding the phage tests) as indicated by Alton *et al.* (1988) and OIE (2008).

For blood culturing, each 5 mL blood sample was added to biphasic medium (trypticase soy solid and liquid phase; Ruiz 1961) and incubated at 37 °C with 5% CO_2 atmosphere for 10 days (Ruiz *et al.* 1997). The solid phase was prepared with 12 mL of trypticase soy agar and the liquid phase consisted of 30 mL trypticase soy broth. Inoculated solid and liquid phase bottles were checked every 24 hours to evaluate haemolysis and turbidity. Once the bacterial growth was detected by turbidity and haemolysis, the colony was sub-cultured and Gram stain was performed to confirm the presence of Gram-negative rods in the broth and on the agar slant. Colonies were stained with Gram and modified Ziehl Neelsen (Stamp's) stains and the reactions to oxidase and catalase were observed (Quinn *et al.* 1994). *Brucella* speciation was done as described for the milk cultures.

Polymerase chain reaction

DNA was extracted from isolates obtained from blood and milk cultures using the Qiagen DNA mini kit (Qiagen) according to the manufacturer's instructions. Each 16-23S ITS PCR (Keid *et al.* 2007) amplification reaction was prepared in a total volume of 25 µL containing 50 mM potassium chloride (KCl), 10 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) (pH 9.0), 2.0 mM magnesium chloride ($MgCl_2$), 200 µM of each deoxynucleotide triphosphates (dNTP), 0.4 µM of each primer, 2.5 µL DNA template and 1.5 U GoTaq® Hot Start Polymerase (Promega). Polymerase chain reaction conditions included an initial denaturation at 95 °C for 5 min followed by 35 cycles consisting of 30 s of denaturation at 95 °C, 30 s of annealing at 56 °C, and 30 s of elongation at 72 °C, with a final elongation at 72 °C for 5 min.

The AMOS-PCR condition was used as previously described by Bricker and Halling (1994, 1995). The PCR reaction consisted of 1.5 mM $MgCl_2$, 1X PCR buffer (Promega), 250 µM dNTPs, 5′ primer cocktail consisting of *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis* specific primers each (0.2 µM) and 1 µM IS711-specific primer, 1 U GoTaq® Hot Start Polymerase (Promega) and 2.5 µL DNA per 25 µL reaction.

All the PCR samples were analysed by electrophoresis in a 2% agarose gel, stained with ethidium bromide (0.5 mg/mL), and the DNA bands were visualised under ultraviolet (UV) light (UVP transilluminator model TM-20). The DNA of *Brucella* reference strains obtained from the *Brucella* culture collection, France (BCCN), namely: *B. abortus* bv 1 (544 = BCCN R4), *B. abortus* bv 2 (86/8/59 = BCCN R5), *B. abortus* bv 4 (292 = BCCN R7), *B. melitensis* (16M = BCCN R1), *B. suis* bv 1 (1330 = BCCN R12), *B. ovis* (63/290 = BCCN R17) and *Brucella canis* (RM6/66 = BCCN R18) were included as positive controls.

Ethical considerations

Research involving animals have been approved by the animal use and care committee of the University of Pretoria and were done according to the national code of welfare standards for each animal species.

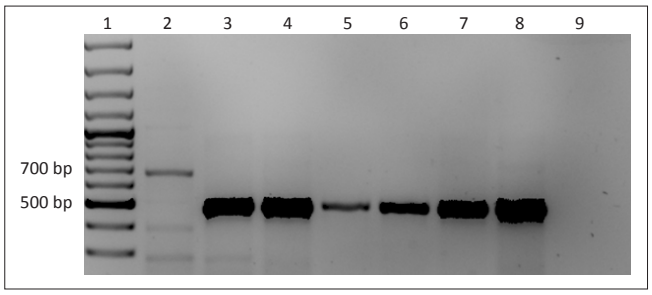
Results

The seroprevalence was 8.3% of the 700 cattle samples from the Malipati and Pesvi in the Chiredzi district based on RBT and iELISA. The prevalence of the individual communal areas were 9% (n = 490) and 6.7% (n = 210) in Malipati and Pesvi, respectively. Table 1 indicates the RBT, cELISA and iELISA serological results of bovine sampled for culturing. *Brucella abortus* was isolated from two seropositive cows in the Malipati and Pesvi regions that were seropositive for RBT, iELISA and cELISA and both had an abortion history (Table 1). The isolates had microscopic and bacteriological characteristics typical of the *Brucella* genus, namely Gram-negative coccobacilli, non-motile, positive for modified Ziehl-Neelsen staining with oxidase and catalase production. Both the *Brucella* cultures could only be identified to species level, namely *B. abortus*, and due to unavailability of phage tests at CVL the biovar(s) could not be determined (Alton *et al.* 1988).

DNA extracted from isolates from blood, milk and *Brucella* reference strains produced a 214 bp product that is specific to *Brucella* using the ITS66 and ITS279 primers for the 16-23S rDNA ITS region (Keid *et al.* 2007). The two isolates from blood and milk identified as *B. abortus* produced the unique 498 bp fragment specific to *B. abortus* bv 1, 2 and 4 using the multiplex AMOS-PCR (Bricker & Halling 1994, 1995; Figure 2).

Discussion

Brucella abortus was isolated and confirmed with AMOS-PCR assay as *B. abortus* bv 1, 2 or 4 strains (Bricker & Halling 1994, 1995) from seropositive cows with an abortion history in the Malipati and Pesvi interface regions in the Chiredzi district in Zimbabwe. These isolates were established from *Brucella* infected cattle samples from the Malipati and Pesvi regions with a seroprevalence of 10.3% (Gomo *et al.* 2012). The *Brucella* seroprevalence rate reported in this study (8.3% of n = 700) using RBT and iELISA was relatively similar to the seroprevalence of 10.3% (n = 1038) using RBT and cELISA reported by Gomo *et al.* (2012). Since none of the sampled



Lane 1, Fermentas GeneRuler 100 bp plus DNA ladder; lane 2, *Brucella melitensis* BCCN R1 (731 bp); lane 3, *Brucella abortus* isolate from animal 10 in Malipati region (498 bp); lane 4, *Brucella abortus* isolate from animal 17 in Pesvi region (498 bp); lane 5-6, *Brucella abortus* bv 1 BCCN R4 (498 bp); lane 7, *Brucella abortus* bv 2 BCCN R7 (498 bp); lane 8, *Brucella abortus* bv 4 BCCN R7 (498 bp); lane 9, negative control.

FIGURE 2: Identification and differentiation of *Brucella abortus* isolated from seropositive cows in the Chiredzi district using automated multiplex oligonucleotide synthesizer multiplex polymerase chain reaction.

cattle had been vaccinated against brucellosis, the detected antibodies were most likely due to a natural infection by *Brucella* species, which was confirmed by cELISA results (Gomo *et al.* 2012) and AMOS-PCR assay as wild-type *B. abortus*. The cELISA and AMOS-PCR differentiate between natural infections and vaccine strains (Bricker & Halling 1994, 1995; Nielsen *et al.* 1989).

Due to the unavailability of phage tests that identify biovars, the *B. abortus* isolates could only be identified to species level using microbiology tests (Alton *et al.* 1988). The AMOS-PCR confirmed the two isolates as *B. abortus* bv 1, 2 or 4 since the multiplex PCR cannot distinguish *B. abortus* bv 1, 2 and 4 from one another (Figure 2; Bricker & Halling 1994, 1995). Previous studies have demonstrated the presence of *B. abortus* bv 1 and 2 in cattle in different parts of Zimbabwe (Madsen 1989; Matope *et al.* 2009; Mohan *et al.* 1996). The majority of the *B. abortus* isolates were found to be biovar 1 (84.6%, 11/13) with the remaining ones being biovar 2 (Matope *et al.* 2009). *Brucella abortus* bv 1 appears to be the predominant cause of brucellosis in cattle in Zimbabwe (Matope *et al.* 2009). Similarly, in neighbouring South Africa, biovar 1 has been shown to contribute about 90% whilst biovar 2 accounted for 10% of all the *B. abortus* isolates (Bishop, Bosman & Herr 1994).

Only two cultures were obtained from milk (n = 10) and blood (n = 18) samples from seropositive cows with an abortion history (7%). Sensitivity of culturing *Brucella* species from blood varies from 10% – 70% of suspected human infections (Al-Attas *et al.* 2000; Pappas *et al.* 2005; Ruiz *et al.* 1997) depending on the growth conditions (Ruiz *et al.* 1997), duration, localisation of the infection and type of *Brucella* species (Al-Attas *et al.* 2000; Ruiz *et al.* 1997). The biphasic method was used to isolate *Brucella* from whole blood samples as described by Ruiz (1961) and incubated for 10 days since Ruiz *et al.* (1997) obtained 100% cultures over a maximum time of 216 hours (9 days). We obtained a low percentage of cultures (7%) from known *Brucella* seropositive samples with unknown stage of brucellosis.

The low sensitivity of culturing *Brucella* in this study clearly indicates that other culturing techniques should be investigated. The lysis centrifugation (LC) technique has been reported by Espinosa *et al.* (2009) to be the preferred technique for *Brucella* culturing at all stages of brucellosis, since it yields 25% more positive results and provided results 10 days earlier than the biphasic method. The LC technique is a yield-optimisation method that uses lysis of erythrocytes in a citrate solution followed by isolation of *Brucella* bacilli by centrifugation that concentrated the bacilli and assist growth (Espinosa *et al.* 2009). The use of the LC technique for *Brucella* culturing with a longer incubation period (40 days [Espinosa *et al.* 2009] compared to 10 days in our study) should rather be used for *Brucella* culturing from blood and milk in future.

Evidence of *Brucella* infections in cattle in the study area has been serologically demonstrated previously (Gomo *et al.* 2012). The isolation of *B. abortus* from *Brucella* seropositive animals confirms the presence of brucellosis and indicates that *B. abortus* might causes abortions in the studied areas since both the cows from which *B. abortus* were isolated had an abortion history. The purchase of unknown *Brucella*-status cattle from the commercial to the communal sector for the purposes of restocking herds and genetic improvements and an increased uncontrolled movement of cattle due to agrarian reforms in the country are reported as the likely source of spread of brucellosis into the communal sector (Matope 2008; Matope *et al.* 2010). In addition, sharing of grazing land and watering points between cattle and wildlife at the studied interface is also likely to be a source of transmission of the disease in both directions as *B. abortus* has been isolated from cattle (Madsen 1989; Matope *et al.* 2009; Mohan *et al.* 1996; this study) and wildlife (Condy & Vickers 1969, 1972). The identification of *B. abortus* known to occur in cattle and wildlife in Zimbabwe is significant since it is one of the species generally considered pathogenic for humans (Baldwin & Goenka 2006). The tradition of consuming unpasteurised milk in rural areas, low awareness of the zoonotic importance of brucellosis, close intimacy with livestock and provision of assistance during parturition may increase the risk of human exposure to *B. abortus* infections in the study area. Despite the prevalence of brucellosis in the study area, no published information is available with regard to human brucellosis. However, public awareness in the Chiredzi communities should be increased to reduce the risk of human exposure to *B. abortus* infection.

Conclusion

Brucella abortus was isolated from blood and milk collected from seropositive cows in the Chiredzi district and therefore the community members in the Chiredzi regions like Pesvi and Malipati should be informed of the risk of human exposure to *Brucella* infection. The isolation of *B. abortus* from seropositive cows confirms that this species could be associated with cattle abortions in the Chiredzi district in Zimbabwe. However, further studies are recommended to determine the distribution of *B. abortus* biovars and human brucellosis prevalence in the area. The need for implementing control measures and raising public awareness on zoonotic transmission of brucellosis is recommended. Serially,

serological testing for brucellosis before translocation, culling of seropositive animals, increased controlled livestock movement and calfhood vaccinations should be instituted for the control of the disease. In addition, simple, user-friendly extension material to make cattle owners aware of this disease and its control should be produced and disseminated to them and the extension staff.

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Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this paper.

Authors' contributions

C.G. (University of Pretoria) and H.v.H. (University of Pretoria) designed the project and wrote the manuscript. C.G. (University of Pretoria) and S.M. (Central Veterinary Laboratory) were involved in morphology identification of the culture and serological testing of the sera. C.G. (University of Pretoria) conducted the molecular studies. M.d.G.-W. (CIRAD), A.C. (CIRAD) and D.M.P. (University of Zimbabwe) were involved with the serological survey and made intellectual and editorial contributions to the manuscript.

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