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Seroprevalence of brucellosis and characterisation of Brucella spp. from slaughtered cattle in Rwanda

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College of Agriculture and Veterinary Medicine,
Department of Veterinary Medicine

December 4th, 2021

To: Editor/ PLOS ONE

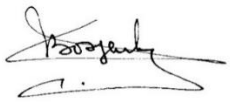
Re: Submission of manuscript

Enclosed is a manuscript to be considered for publication in PLOS ONE. The title of the manuscript is "seroprevalence of brucellosis and molecular characterization of *Brucella* spp. from slaughtered cattle in Rwanda". Bovine brucellosis is a zoonotic disease that is prevalent in the cattle population in Rwanda. However, there is not sufficient data about the seroprevalence and *Brucella* spp. circulating in Rwandan cattle. We investigated the seroprevalence of brucellosis and characterized *Brucella* spp. from slaughtered cattle in Rwanda. Our findings demonstrated the overall seroprevalence of 2.9% (8/300) using the Rose Bengal Test and indirect ELISA in parallel. The culture prevalence confirmed with *Brucella* specific 16S-23S ribosomal DNA interspace region (ITS) PCR was 5.6% (17/300). AMOS-PCR assay identified mixed *B. abortus* and *B. melitensis* (n=3), *B. abortus* (n=3) and *B. melitensis* (n=5) while Bruce-ladder PCR confirmed *B. abortus* (n=5) and *B. melitensis* (n=6). The gold standard culture method combined with PCR confirmation (5.6%) was higher than the more sensitive seroprevalence of 2.9%. This emphasizes the need to validate the serological tests in Rwanda. The mixed infection caused by *B. abortus* and *B. melitensis* in slaughtered cattle indicates cross-infection and poses a risk of exposure potential to abattoir workers. It is essential to urgently strengthen the national bovine brucellosis control program through vaccination as well as test-and-slaughter.

This manuscript is an original work that meets the scope and aims of the PLOS ONE and has not been submitted to any journal for publication.

I am Jean Bosco Ntivuguruzwa, a corresponding author on behalf of all other co-authors.

Yours sincerely



Jean Bosco Ntivuguruzwa, the corresponding Author

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Seroprevalence of brucellosis and molecular characterization of *Brucella* spp. from slaughtered cattle in Rwanda

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Abstract

Bovine brucellosis is endemic in Rwanda, although, there is paucity of documented evidence about the disease in slaughtered cattle. A cross-sectional study was conducted in slaughtered cattle (n=300) to determine the seroprevalence of anti-*Brucella* antibodies using the Rose Bengal Test (RBT), and indirect enzyme-linked immunosorbent assay (i-ELISA). Corresponding tissues were cultured onto a modified Centro de Investigación y Tecnología Agroalimentaria (CITA) selective medium and analysed for *Brucella* spp. using the 16S-23S ribosomal interspacer region (ITS), AMOS, and Bruce-ladder PCR assays. The RBT seroprevalence was 20.7% (62/300), and 2.9% (8/300) with i-ELISA and 2.9% (8/300) using both tests in parallel. *Brucella* specific 16S-23S ribosomal DNA interspace region (ITS) PCR detected *Brucella* DNA in 5.6% (17/300; *Brucella* culture prevalence). AMOS-PCR assay identified mixed *B. abortus* and *B. melitensis* (n=3), *B. abortus* (n=3) and *B. melitensis* (n=5) while Bruce-ladder PCR also identified *B. abortus* (n=5) and *B. melitensis* (n=6). The gold standard culture method combined with PCR confirmation identified 5.6% *Brucella* cultures which is higher than the more sensitive seroprevalence of 2.9%. This emphasizes the need to validate the serological tests in Rwanda. The mixed infection caused by *B. abortus* and *B. melitensis* in slaughtered cattle indicates cross-infection and poses a risk of exposure potential to abattoir workers. It is essential to urgently strengthen the national bovine brucellosis control program through vaccination as well as test-and-slaughter.

Keywords

Abattoirs; *Brucella* spp.; cattle; culture; PCR; serology; Rwanda

Introduction

Brucellosis is a contagious widespread disease that causes not only substantial economic losses related to abortions, long conception intervals, and sterility in animals but also morbidity and reduced working capacity in humans (1, 2). The disease is caused by bacteria of the genus *Brucella* which belongs to the family of alphaproteobacteria (3). *Brucella* species are gram-negative microaerophilic coccobacilli, acid-fast intracellular, and hosts-specific microorganisms affecting a wide variety of terrestrial and marine mammals (4, 5). *Brucella* species are 96% genetically identical (6) with few polymorphisms that are essential for species and biovars differentiation (7, 8). Classical species with their biovars (bv.) have specific hosts, for instance, *B. abortus* (7 biovars) infect primarily cattle, *B. melitensis* (3 biovars) infect goats and sheep, *B. ovis* infect sheep, *B. suis* (bv. 1, 3, 4, and 5) infect swine while *B. suis* bv. 2 infects rats, *B. canis* infects dogs, and *B. neotomae* infects wood rats (4, 9).

The transmission of brucellosis in animals is through inhalation of *Brucella* aerosols (10), direct contact with infective foetal membranes, vaginal discharges, placenta content, and ingestion of contaminated feeds (11). There are no pathognomonic clinical signs for brucellosis, but cases of abortion or hygroma are suspicious signs that require laboratory diagnosis for confirmation (9, 12).

As brucellosis is a herd disease, the most suitable tests are serological tests to determine the seroprevalence of brucellosis in the animal and or herd using a screening agglutination test, the Rose Bengal Test (RBT), and a confirmatory test like enzyme-linked immunosorbent assays

59 (ELISAs) or complement fixation test (CFT) (9). However, serological tests do not provide a
60 complete diagnosis, thus, the isolation of *Brucella* spp. remains the gold standard (9). The
61 culturing and biotyping of *Brucella* cultures are expensive, time-consuming, and require trained
62 personnel (7). PCR assays which differentiate *B. abortus* bv.1, 2, 4, *B. melitensis* bv.1, 2, 3, *B. ovis*,
63 and *B. suis* bv.1 (AMOS PCR) in 24 hours from cultures (7) and Bruce-ladder PCR assay can
64 differentiate all *Brucella* species and vaccine strains (13, 14). Unfortunately, culture, phenotypic
65 and genotypic isolation of *Brucella* spp. are not common in veterinary services in most developing
66 countries owing to inadequate facilities and trained personnel; therefore, serology is in common
67 practice with little knowledge on the type of infecting *Brucella* spp. (15).

68 Brucellosis is an endemic disease in Rwanda with a reported 7.4% to 18.7% seroprevalence
69 in cattle (16, 17), as well as seroprevalence in women with a history of abortions varying between
70 6.1% and 25.0% (18, 19). Although, cattle from various districts of the country are slaughtered at
71 abattoirs, there is no single study on the seroprevalence of brucellosis in slaughtered cattle in
72 Rwanda. Furthermore, apart from a single study that isolated *B. abortus* bv. 3 from Rwandan cattle
73 in the 1980s (20), *Brucella* spp. that are circulating in Rwanda are not known. The objective of this
74 study was, therefore, to determine the seroprevalence of brucellosis and characterize *Brucella* spp.
75 from slaughtered cattle in Rwanda. Our findings are essential to building an epidemiological
76 database essential for the control of brucellosis in Rwanda.

Materials and methods

Study area

This study was conducted at six abattoirs in Rwanda. Rwanda is a landlocked country of the East African community covering an area of 26,338 Km^2 in the southern hemisphere near the equator (West: 28.86; East: 30.89; North: - 1.04; South: - 2.83). The bovine population in Rwanda was estimated at 1,293,768 in 2018 (21). The six abattoirs (société des abattoirs de Nyabugogo "SABAN", Rugano abattoir, Kamembe, Rubavu, Kamuhanda, Gataraga) consented to participate (Fig 1). These abattoirs were selected based on their slaughtering capacity and their location to sample cattle from all the thirty districts of Rwanda. In this study, cattle sampled at SABAN abattoir were from 19 districts including Rulindo, Ngoma, Muhanga, Nyagatare, Gasabo, Bugesera, Ngororero, Gakenke, Burera, Rutsiro, Gicumbi, Nyarugenge, Kirehe, Ruhango, Kayonza, Karongi, Nyanza, Kamonyi, and Gatsibo. Cattle sampled at Rugano abattoir were from three districts including Gasabo, Rwamagana, and Nyarugenge. Cattle sampled at Kamembe abattoir were from eight districts including Gisagara, Huye, Nyamagabe, Nyamasheke, Nyanza, Nyaruguru, Ruhango, and Rusizi. Cattle sampled at Rubavu abattoir were from two districts including Nyabihu, and Rubavu. Cattle sampled at Kamuhanda abattoir were from the Kamonyi district. Cattle sampled at Gataraga abattoir were from the Musanze district. These abattoirs were classified into high throughput abattoirs (n = 4) slaughtering more than 50 cattle daily and low throughput abattoirs (n = 2) slaughtering 50 or less every day.

Fig 1. Map of provinces and districts of Rwanda with location (red asterisks) of abattoirs visited during this study.

Study design and sample size

A cross-sectional study was carried out from August 2018 through October 2019 to determine the seroprevalence of brucellosis and characterize *Brucella* spp. from cattle tissue selected during slaughtering at abattoirs. The sample size was calculated using the previously described formula (22) for cross-sectional studies.

$$N = \frac{Z^2 \quad P(1-P)}{d^2}$$

Where N is the sample size, $Z^2 = 1.96$ the statistical constant at 95% confidence interval; P is the expected prevalence and was estimated at 0.5%, and the absolute precision, d = (P/2). According to the formula, the total sample size was 291 but it was rounded to 300 cattle to sample ten animals per each of the 30 districts of Rwanda.

Sampling procedure

Our target was to sample five animals coming from the same district every day. The district of origin of animals was recorded on arrival using the movement permit issued by the sector animal resources officer at the animal market. The age was determined using teeth erosion as previously described (23). Except for abattoirs that received mostly males, females of one year and above were selected using a systematic random sampling. Animals were aligned in a crush

114 and every fourth animal was selected for sampling. The vaccination status and farm of origin of
115 slaughtered animals could not be traced because most of the animals were bought from the
116 different animal markets.

117 Collection of blood and tissues samples

118 After the selection and recording of individual demographic information (district of
119 origin, age, breed, and sex), the animal was restrained, marked on the head, and released for
120 resting waiting for the collection of blood after bleeding. Blood was collected into sterile 50 ml
121 tubes after slaughter, aliquoted into 5 ml tubes and was immediately transported to the
122 laboratory of the University of Rwanda (UR) and left overnight at room temperature to allow
123 clotting. The following day, serum was collected into a sterile 2 ml micro-centrifuge tube and
124 stored at -20°C until serological testing at Rwanda Agriculture and Animal Resources Board
125 (RAB), Department of Veterinary Services, in the serology section. The head of the marked animal
126 from which blood was collected was followed at head inspection station and the corresponding
127 left and right retropharyngeal lymph nodes were collected into a sterile 50 ml tube.

128 Serological tests

129 Animal sera were tested with the RBT following the manufacturer's guidelines (IDvet,
130 France) and the OIE protocol (9). Briefly, equal volumes (30 µl) of *Brucella* antigens and sera were
131 gently mixed for four minutes, and any agglutination was regarded as a positive result. All sera
132 were also checked for the presence of anti-*Brucella* antibodies with a confirmatory test kit namely

133 a multispecies i-ELISA according to manufacturer's guidelines (IDvet, France) and the OIE
134 protocol (9) with positive and negative controls. The cut-off point for the seropositivity was 120%
135 and sera samples having 120% of optical densities were considered positive. These serological
136 tests were chosen because of their combined effects of sensitivity and specificity (24). RBT is a
137 screening test with high sensitivity, while i-ELISA is a confirmatory test with high specificity (24,
138 25). Any detection of anti-*Brucella* antibodies by RBT or i-ELISA was considered to determine the
139 seroprevalence of brucellosis.

140 Culturing and *Brucella* isolation from tissues

141 Tissue samples were processed and cultured in a biosafety level 3 (BSL 3) facility at the
142 National Reference Laboratory (NRL), Rwanda Biomedical Centre (RBC), Kigali, Rwanda
143 according to the guidelines previously described (9). Briefly, tissues were sliced using sterile
144 scissors and forceps into sterile mortars and grounded using a sterile pestle. An aliquot of pooled
145 homogenate was spread into a modified Centro de Investigación y Tecnología Agroalimentaria
146 (CITA) selective medium and incubated at 37°C with 10.0% CO_2 atmosphere (26). Plates were
147 read for bacterial growth every day for four weeks. The DNA was extracted from colonies suspect
148 of *Brucella* organisms.

149 DNA extraction and identification of the genus *Brucella* spp.

150 Genomic DNA was extracted from cultures using the ReliaPrep gDNA tissue Miniprep
151 system following the manufacturer's guidelines (Promega, USA). This DNA was screened for

152 *Brucella* DNA using *Brucella* specific primers (Table 1) designed from a gene-specific 16S -23S
153 rDNA interspacer region (ITS) (27) with the *B. abortus* RF 544 (Onderstepoort Biological Products,
154 South Africa) as a positive control. The 15 µl PCR reaction mixture contained 1x of MyTaq™ Red
155 PCR Mix (Bioline, South Africa), primers at 0.2 µM, and 2 µl of template DNA. The PCR cycling
156 condition was initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation at 95°C
157 for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 2 min and a final extension step
158 at 72°C for 5 min. The primers amplified a 214 bp fragment that was analyzed by electrophoresis
159 using a 2% agarose gel stained with red gel nucleic acid stain and visualized under UV light.
160 Molecular analyses were done at the Department of Veterinary Services, Rwanda Agriculture
161 Board (RAB) Kigali, Rwanda.

162 Identification of *Brucella* species using AMOS and Bruce-ladder PCR
163 assays

164 The DNA samples that were ITS PCR positive were tested for *B. abortus*, *B. melitensis*, *B.*
165 *ovis*, and *B. suis* using a multiplex AMOS PCR assay as previously described (7). A 25 µl reaction
166 mixture contained 1x MyTaq Red PCR Mix (Bioline, South Africa), four species-specific forward
167 primers and reverse primer IS711 (Table 1) at a final concentration of 0.1 µM and 0.5 µM
168 respectively, and 2 µl of template DNA. Thermocycling conditions included initial denaturation
169 at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 2
170 min, an initial extension at 72°C for 2 min, and a final extension at 72°C for 5 min. PCR products

171 were analysed by gel electrophoresis using 2% agarose stained with red gel nucleic acid stain and
172 visualized under UV light.

173 Vaccine strains and field isolates of *Brucella* spp. were identified and differentiated by a
174 multiplex Bruce-ladder PCR as previously described (14). A 25 µl PCR reaction contained 1x
175 MyTaq™ Red Mix (Bioline, South Africa), eight species-specific forward and reverse primers at a
176 final concentration of 6.25 µM (Table 1), and 5 µl of template DNA. The PCR cycling conditions
177 included an initial denaturation at 95°C for 5 min followed by 25 cycles of at 95°C for 30 s, at 64°C
178 for 45 s, and at 72°C for 3 min and a final extension step at 72°C for 10 min. PCR products were
179 analysed by gel electrophoresis using a 2% agarose stained with gel red nucleic acid stain and
180 viewed under UV light.

181 Data analysis

182 The overall seroprevalence was obtained by dividing the total number of animals
183 simultaneously positive to RBT and i-ELISA by the total number of animals sampled. Data were
184 recorded in Microsoft Excel spreadsheets. Epi-Info 7 version 10 was used to calculate proportions.
185 Significant levels between individual risk factors and seroprevalence and molecular results were
186 determined using the chi-square test. The odds-ratios were determined for associated risk factors
187 along 95% confidence intervals and statistical significance set at $p < 0.05$.

188 Ethics clearance

189 The authorization to conduct the study was obtained from the research screening and
190 ethical clearance committee of the College of Agriculture, Animal Sciences and Veterinary
191 Medicine, University of Rwanda (Ref:026/DRIPGS/2017), institutional review board of the
192 College of Medicine and Health Sciences, University of Rwanda (N° 006/CMHS IRB/2020), and
193 Animal Ethics Committee of the Faculty of Veterinary Science, University of Pretoria, South
194 Africa (V004/2020). Informed verbal consent was obtained from managers of abattoirs, and
195 owners of animals at the abattoirs.

196 Table 1: Sequences of oligonucleotide primers used for the distinction of *Brucella* species isolated from slaughtered cattle in Rwanda

197

PCR name	Primer name	Sequence (5'-3')	Targets	Size (bp)	Conc. (µM)	References
ITS	ITS66 f	ACATAGATCGCAGGCCAGTCA	*16s-23s rDNA*	214	0.2	(27)
	ITS279 r	ACATAGATCGCAGGCCAGTCA				
AMOS	*B. abortus*	GAC GAA CGG AAT TTT TCC AAT CCC	IS711	498	0.1	(7)
	B. melitensis	AAA TCG CGT CCT TGC TGG TCT GA		731	0.1	
	B. ovis	CGG GTT CTG GCA CCA TCG TCG GG		976	0.1	
	B. suis	GCG CGG TTT TCT GAA GGT GGT TCA		285	0.1	
	IS 711	TGC CGA TCA CTT AAG GGC CTT CAT			0.2	
BRUCE- LADDER	BMEI0998f	ATC CTA TTG CCC CGA TAA GG	*wboA*	1682	6.25	(28, 29)
	BMEI0997r	GCT TCG CAT TTT CAC TGT AGC				
	BMEI0535f	GCG CAT TCT TCG GTT ATG AA	*bp26*	450	6.25	(30)
	BMEI0536r	CGC AGG CGA AAA CAG CTA TAA				
	BMEII0843f	TTT ACA CAG GCA ATC CAG CA	*omp31*	1071	6.25	(31)
	BMEII0844r	GCG TCC AGT TGT TGT TGA TG				
	BMEI1436f	ACG CAG ACG ACC TTC GGT AT	*Deacetylase*	794	6.25	(32)
	BMEI1435r	TTT ATC CAT CGC CCT GTC AC				
	BMEII0428f	GCC GCT ATT ATG TGG ACT GG	*eryC*	587	6.25	(33)
	BMEII0428r	AAT GAC TTC ACG GTC GTTCG				
	BR0953f	GGA ACA CTA CGC CAC CTT GT	*ABC Transporter*	272	6.25	(34)
	BR0953r	GAT GGA GCA AAC GCT GAA G				
	BMEI0752f	CAG GCA AAC CCT CAG AAG C	*rpsL*	218	6.25	(35)
	BMEI0752r	GAT GTG GTA ACG CAC ACC AA				
	BMEII0987f	CGC AGA CAG TGA CCA TCA AA	*CRP Regulator*	152	6.25	(32)
	BMEII0987r	GTA TTC AGC CCC CGT TAC CT				

198

Results

Brucellosis seroprevalence among slaughter cattle in Rwanda

Of the 300 cattle sera, 95.7% (287/300) were from females, while 4.3% (13/300) were from males. Most animals, 89.7% (269/300) were adults while young animals represented 10.3% (31/300). Twenty-seven percent [27.7%, (81/300)] of cattle sampled were local breed "Ankole", 67.0% (201/300) were crossbreeds and 5.3% (16/300) were Friesians. Samples were mainly collected from high throughput abattoirs (n=280) compared to low throughput abattoirs (n=20). The seroprevalence of brucellosis in parallel was 20.7% (62/300) and 2.9% (8/300) using RBT and i-ELISA, respectively. The seroprevalence was 2.9%, (8/300) using both tests in parallel. Twenty-one percent [21.1%, (59/280)] of the RBT positive cattle were sampled from high throughput abattoirs, while 15.0% (3/20) were from the low throughput abattoirs. Eight samples that were simultaneously positive on RBT and i-ELISA were collected from high throughput abattoirs (Table 2). Considering the RBT results alone, the highest RBT positivity rate of 30.0%, (21/70) was observed in the Eastern Province while the lowest 13.3%, (4/30) was recorded in the Kigali city (Table 2). Twenty-one percent [21.6%, (58/269)] of RBT seropositive animals were adult, while 12.9%, (4/31) were young. Twenty percent [20.6%, (59/287)] of RBT seropositive animals were female while 23.1%, (3/13) were young. The eight animals that were seropositive to both tests were adult females. The RBT seropositivity recorded in different breeds of animals was as follows: 15.7%, (13/83) for Ankole, 22.0%, (44/201) for crossbreds and 31.3%, (5/16) for Friesians with no significant statistical difference ($p = 0.28$). However, there was a significant association (p

219 = 0.04) between seropositivity (RBT and i-ELISA) and breeds with Friesian being more

220 seropositive 12.5%, (2/16) than crossbreds 2.5%, (5/201) and Ankole 1.2%, (1/83) (Table 1).

221 Table 2: Univariate associations between animal characteristics, seropositivity, and isolation of *Brucella* spp. using ITS PCR assay on
222 the DNA extracted from cultures of tissues of slaughtered cattle in Rwanda.

Variables	Categories	Tested	RBT			i-ELISA			ITS PCR assay on culture isolates		
			n^+ (%)	OR	*p*-value	n^+ (%)	OR	*p*-value	n^+/N (%)	OR	*p*-value
Abattoirs	High thr.	280	59 (21.1)	0.8 – 1.1	0.78	8 (2.9)	0.1-0.2	1	16/78 (20.5)	0.69-1.16	0.68
	Low thr.	20	3 (15.0)			0 (0.0)			1/9 (11.1)		
Provinces	Eastern	70	21 (30.0)	-	0.17	3 (4.3)	-	0.37	2/14 (14.3)	-	0.95
	Kigali city	30	4 (13.3)			3 (6.0)			3/11 (27.3)		
	Northern	50	7 (14.0)			1 (1.3)			4/18 (22.2)		
	Southern	80	14 (17.5)			1 (1.4)			3/17 (17.6)		
	Western	70	16 (22.7)			1 (1.4)			5/27 (18.5)		
Age	Adults	269	58 (21.6)	0.8 – 1.0	0.35	8 (3.0)	0.95-0.99	1	17/77 (22.1)	0.69-0.88	0.19
	Young	31	4 (12.9)			0 (0.0)			0/10 (0.0)		
Sex	Females	287	59 (20.6)	0.8– 1.4	0.74	8 (2.8)	0.95-0.99	1	17/80 (21.3)	0.70-0.88	0.34
	Males	13	3 (23.1)			0 (0.0)			0/7 (0.0)		
Breed	Ankole	83	13 (15.7)	-	0.28	1 (1.2)	-	0.04	1/21 (4.8)	-	0.02
	Cross	201	44 (22.0)			5 (2.5)			13/61 (21.3)		
	Friesian	16	5 (31.3)			2 (12.5)			3/5 (60.0)		

223 RBT: Rose Bengal Test, i-ELISA: indirect enzyme-linked immunosorbent assay, Thr. = throughput; OR = odds ratio; CI = confidence
224 interval; n+: number of positives; n+/N: number of positive animals over the total number of tested animals; %: percentage, NaN: not
225 applicable.

226

227 ## Bacteriology and the 16S-23S interspacer region (ITS) PCR assay

228 Of the tissues (n=300) that were cultured onto the modified CITA medium, ITS PCR
229 confirmed 5.6% (17/300) (Fig 2). Therefore, the prevalence obtained by bacteriology, the gold
230 standard and confirmed by ITS PCR was 5.6% (17/300).

231 Fig 2. Agarose gel electrophoresis of the 16-23S interspacer region (ITS) PCR products amplified
232 from cultures of tissues from slaughtered cattle. Lanes M: DNA Gene Ruler 100bp plus
233 (Invitrogen, Pretoria, South Africa), lanes 1 – 7: amplification of a 214 bp sequence of the genus
234 *Brucella* spp., lane 8: negative control containing sterile water, lane 9: positive control with *B.*
235 *abortus* RF544.

236 ## Differentiation of *Brucella* spp. by AMOS and Bruce-ladder PCR assays

237 The AMOS PCR identified *B. melitensis* and *B. abortus* (n=3) mixed cultures, *B. abortus* (n=3)
238 and *B. melitensis* (n=5) (Fig 3) from the 17 *Brucella* cultures (impure culture). The Bruce-ladder
239 PCR assay identified *B. abortus* (n=5), *B. melitensis* (n=6) (Fig 4).

240 Fig 3. Agarose gel electrophoresis for AMOS PCR products amplified from cultures of tissues
241 from slaughtered cattle. Lanes M: Gene Ruler 100 pb plus (Invitrogen, ThermoFischer, South
242 Africa), lanes 1-4: *Brucella abortus* (496 bp), lanes 5-7: *B. melitensis* (731 bp), Lanes 9-10: mixed *B.*
243 *melitensis* and *B. abortus*, lane 11: negative control containing sterile water, lane 12: positive
244 control, *B. abortus* RF544, lane 13: positive control, *B. melitensis* rev 1.

245 Fig 4. Agarose gel electrophoresis for Bruce–ladder PCR products amplified from cultures of
246 tissues from slaughtered cattle. Lanes M: Gene Ruler 100 bp (Invitrogen, ThermoFischer, South
247 Africa); lanes 1-5: *B. abortus*; lanes 6-8: *B. melitensis*; lane 9: positive control, *B. suis* ZW45, lane 10:
248 positive control, *B. melitensis* rev 1, lane 11: *B. abortus* (REF 544), lane 12: positive control, *B. abortus*
249 S 19, lane 13: negative control with sterile water.

250 Culture prevalence amongst slaughtered cattle in Rwanda

251 The *Brucella* culture prevalence obtained by culture and confirmed by ITS PCR was 5.6%
252 (17/300). Of the *Brucella* spp. obtained by culture, 20.5% (16/78) were collected from high
253 throughput abattoirs, while 11.1% (1/9) were from low throughput abattoirs (OR = 0.69 – 1.16, p
254 = 0.68). There was no significant association between the isolation of *Brucella* spp. isolates and the
255 provinces of origin (p = 0.95), and the isolates were collected from the provinces as follows:
256 Eastern (14.3%, 2/14), Kigali city (27.3%, 3/11), Northern (22.2%, 4/18), Southern (17.6%, 3/17),
257 Western (18.5%, 5/27). All the *Brucella* obtained by culture (21.3%, 17/80) were adult (OR = 0.69 -
258 0.88, p = 0.20) and females (OR = 0.70 - 0.88, p = 0.34). There was a significant association between
259 the culture prevalence and breed (p = 0.02) with Friesians having more isolates (60.0%, 3/5),
260 followed by crossbreds (21.3%, 13/61) and Ankole (4.8%, 1/21) (Table 2).

261 The *Brucella* DNA detected by ITS, AMOS and Bruce-ladder PCR assays (100.0%,
262 11/11) were from cattle that were either seropositive to RBT or i-ELISA. Of these 11 *Brucella*
263 isolates, 10 were isolated from slaughtered cattle collected at high throughput abattoir.
264 The 11 *Brucella* isolates that were identified in provinces are as follows: Eastern (n = 1),

265 Kigali city (n = 2), Southern (n = 3), Western (n = 2), Northern (n = 3). The 11 *Brucella*
266 isolates stratified by breeds were Ankole (n = 1), crossbreds (n = 8) and Friesians (n = 2).
267 There was no significant difference between the category of abattoirs, provinces, age, sex
268 of animals and the detection by ITS, AMOS and Bruce-ladder PCR assays.

Discussion

This is the first report of *B. abortus* and *B. melitensis* isolated from cultures of cattle tissues collected from abattoirs. The overall seroprevalence obtained in this study among slaughtered cattle selected from all the thirty districts of Rwanda (2.9% for RBT and i-ELISA) was lower than the culture prevalence of 5.6% (17/300), which is the gold standard. The fact that the lower sensitivity culture method is higher than the seroprevalence is a clear indication that the confirmatory i-ELISA test must be validated for bovine in Rwanda as the cut-off values were determined in developed countries with low brucellosis prevalence and thus clearly underestimate the prevalence due to high cut-off values.

The overall seroprevalence of 2.9% was also lower than the rates reported in Rwanda in different studies that were conducted at farm level (16, 17, 36). However, the seroprevalence obtained in this study is comparable with the rate (3.4%) reported at Gaoundere municipal abattoir in Cameroun using RBT and i-ELISA (37), and the 3.9% reported among slaughtered cattle in Nigeria (38), and the 5.5% reported among slaughtered cattle in Gauteng province, South Africa (39).This suggests that the seroprevalence rates observed in abattoirs are usually lower compared to the seroprevalence recorded at the farm level which usually focuses on endemic zones while slaughtered cattle come from various locations (endemic and non-endemic zones).

Friesians were more likely to be seropositive in this study and was consistent with earlier studies in Pakistan where Holstein and Friesian cattle were more seropositive than indigenous breeds (40), and in Ethiopia (41). This supports that exotic pure breeds like Friesians are more susceptible to brucellosis than crossbreeds and indigenous breeds (42) or were introduced in the

290 herd with chronic infections with seronegative status but being chronically infected (12, 43).
291 When the acute brucellosis phase has passed, the infection stabilizes with the acquisition of herd
292 immunity leading to less infectious discharges and non-visible symptoms (44).

293 The mixed infection caused by *B. abortus* and *B. melitensis* and the isolation of *B. melitensis*
294 from slaughtered cattle indicate the cross-infection between both *Brucella* spp. and mixed farming
295 of cattle and goats or sheep. The mixed infection and mixed farming were reported in our study
296 that identified both pathogens in aborting goat flock in Rwanda (unpublished data). The co-
297 infection of *B. abortus* and *B. melitensis* has also been reported in slaughtered cattle in South Africa
298 (39). The isolation of *B. melitensis* in slaughtered cattle poses a risk to abattoir workers and
299 consumers of of contaminated milk and milk products as *B. melitensis* and *B. abortus* cause severe
300 brucellosis in humans (45, 46). There is a need for improvement in brucellosis control using
301 vaccination as well as test-and-slaughter, coupled with raising awareness of all occupational
302 groups as education was associated with a high awareness of brucellosis in Rwanda (17).

303 Both AMOS and Bruce-ladder PCR assays identified *B. abortus* and *B. melitensis* with the
304 *B. abortus* being either biovars 1, 2, or 4 (identified by AMOS PCR) which will be identified in the
305 future after purification of cultures using biotyping. A previous study *B. abortus* bv. 3 was
306 identified in humans and animals in 1987 in Rwanda (20). *B. abortus* bv. 3 and *B. melitensis* bv. 1
307 were reported in neighboring Uganda (47), Tanzania (48), Kenya (49) and South Africa (39).
308 Biotyping of *B. abortus* biovars is complex as characteristic typical for *B. abortus* bv .1, except CO_2
309 requirement for growth (50). However, the *B. abortus* bv. 3 ref strain Tulya isolated from human
310 patient in Uganda grows in the absence of CO_2 and has been observed to occur within some

311 biovars and changes with OIE biotyping profile (9, 48). Hence classifying *B. abortus* bv. 3 strains
312 should be carefully considered. Purifying and biotyping these cultures will be able to identify the
313 biovar(s) and molecular characterization of the strains will allow trace back studies. *Brucella*
314 *abortus* and *B. melitensis* isolated in this study could originate from neighboring countries due to
315 repatriation of Rwandans and their livestock from Uganda and Tanzania as well as importation
316 of improved cattle breeds from various countries cannot be eliminated despites testing
317 procedures (12).

318 *Brucella* spp. were mostly isolated from adult females, and this is not surprising as cattle
319 industry focuses mostly on dairy production while commercial beef production is emerging in
320 Rwanda. Almost half (47.0%) of the milk produced in 2008 was destined for sale at the informal
321 market, with 16.0% for home consumption, while 35.0% represented spoiled milk (51). Therefore,
322 the occurrence of brucellosis in slaughtered cattle is not only a risk to abattoir workers but also
323 consumers of milk and milk products. Several cattle and tons of beef are sold to Bukavu and
324 Goma, the towns of the Democratic Republic of Congo neighbouring Rwanda where the
325 brochettes of the udder are expensive and frequently consumed. The udder is among the
326 predilection sites of *Brucella* spp. (52, 53) and meat inspection should focus on the udder. Meat
327 inspection provides safe meat and contributes to the monitoring and surveillance for animal
328 infectious diseases and zoonoses (54). Furthermore, these brochettes should be consumed well
329 done. It is also important to raise the awareness of involved stakeholders through education
330 campaigns or media.

Conclusions

This study found the seroprevalence of brucellosis to be lower than the gold standard rate indicating that cut-off points of i-ELISA determined in Europe with brucellosis free status or low prevalence, should be optimized for Rwanda as also reported by Mathew *et al.* (2015). This study identified *B. abortus* and *B. melitensis* as well as mixed infection in slaughtered cattle which is as a result of the mixed livestock farming practice in Rwanda. These infections pose a risk of exposure potential to handlers of cattle, carcasses and consumers of unpasteurized milk and milk products. Thus, vaccination and test-and-slaughter would significantly contribute to mitigate the disease. Furthermore, the introduction of an annual brucellosis-free certificate for large herds would contribute to mitigating brucellosis in the country.

Author's contributions

Conceptualization, JBN, and HvH; methodology, JBN, and HvH; Formal analysis JBN; Investigation and data collection, JBN; Writing—original draft preparation, JBN; writing—review and editing, JBN, IEM, FBK, and HvH; Supervision, HvH, and FBK; Project administration, HvH; Resources, HvH; Funding acquisition, HvH. All authors have read and approved the manuscript.

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357 Conflict of interest

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361 Availability of Data

362 All data will be available in the supplementary material of this journal.

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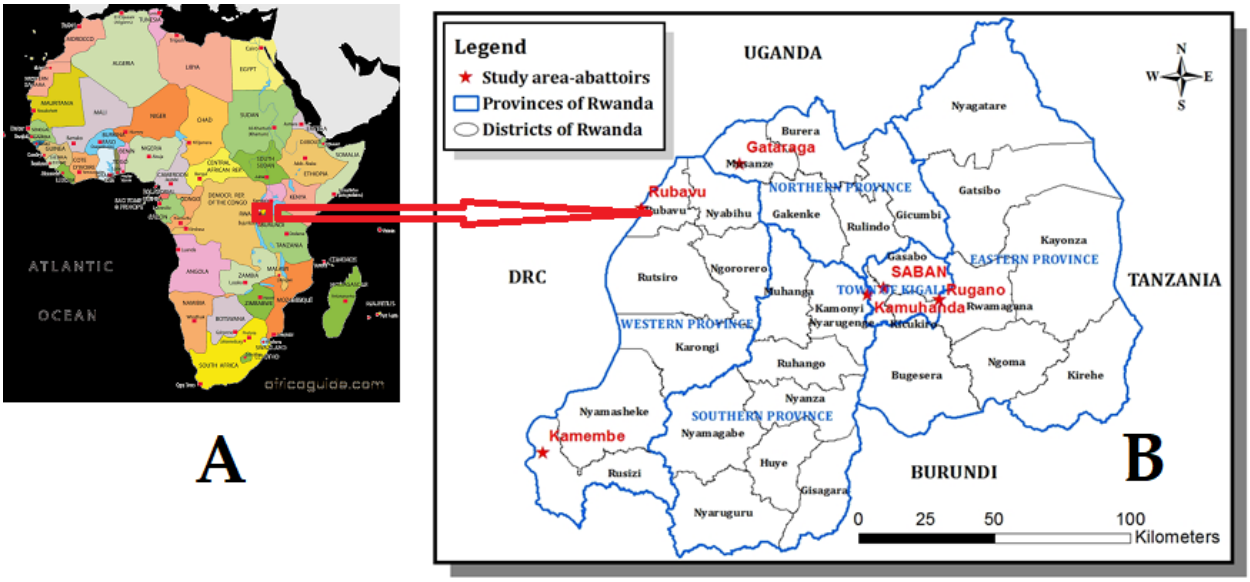

Fig 1. Map of provinces and districts of Rwanda with location (red asterisks) of abattoirs visited during this study

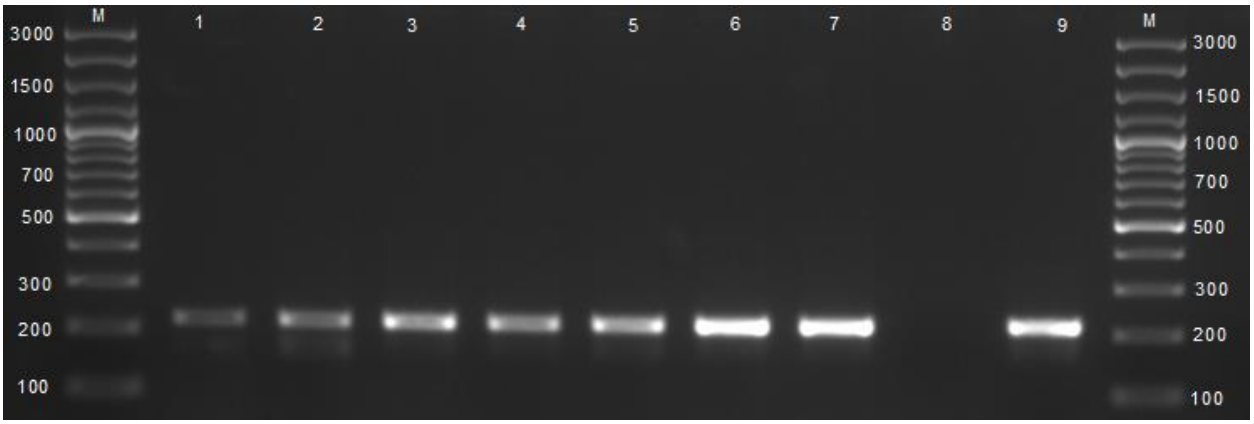

Fig 2. Agarose gel electrophoresis of the 16-23S interspacer region (ITS) PCR products amplified from cultures of tissues from slaughtered cattle. Lanes M: DNA GeneRuler 100bp plus (Invitrogen, Pretoria, South Africa), lanes 1 – 7: amplification of a 214 bp sequence of the genus *Brucella* spp., lane 8: negative control containing sterile water, lane 9: positive control with *B. abortus* RF544.

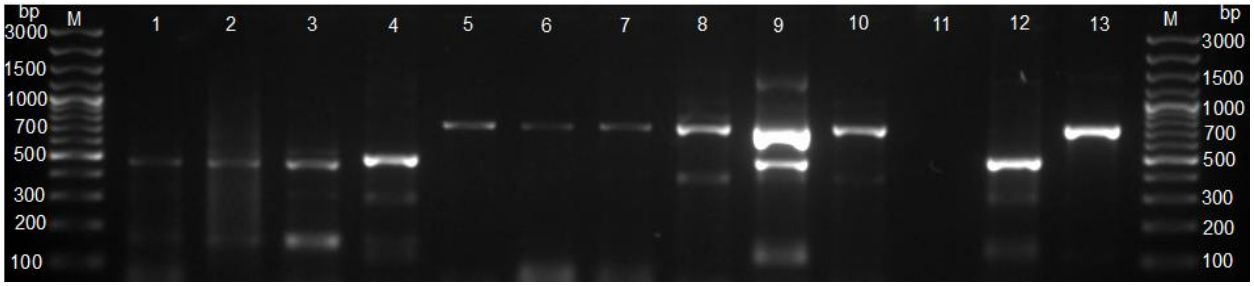

Fig 3. Agarose gel electrophoresis for AMOS PCR products amplified from cultures of tissues from slaughtered cattle. Lanes M: Gene Ruler 100 pb plus (Invitrogen, ThermoFischer, South Africa), lanes 1-4: *Brucella abortus* (496 bp), lanes 5-7: *B. melitensis* (731 bp), Lanes 9-10: mixed *B. melitensis* and *B. abortus*, lane 11: negative control containing sterile water, lane 12: positive control, *B. abortus* RF544, lane 13: positive control, *B. melitensis* rev 1.

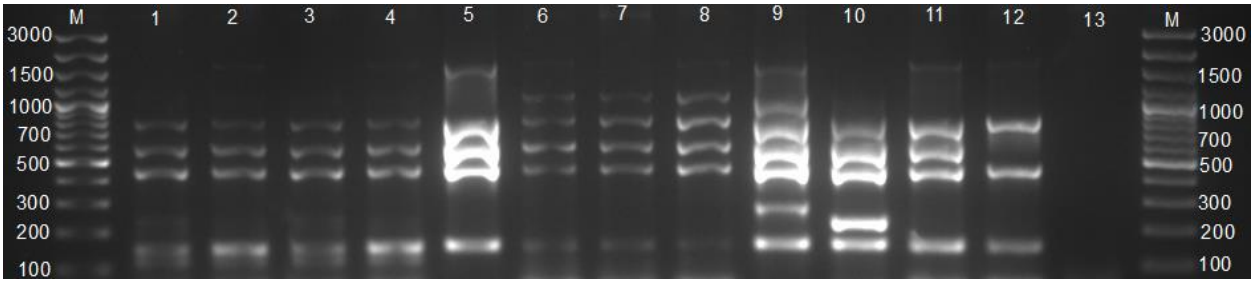

Fig 4. Agarose gel electrophoresis for Bruce–ladder PCR products amplified from cultures of tissues from slaughtered cattle. Lanes M: Gene Ruler 100 bp (Invitrogen, ThermoFischer, South Africa); lanes 1-5: *B. abortus*; lanes 6-8: *B. melitensis*; lane 9: positive control, *B. suis* ZW45, lane 10: positive control, *B. melitensis* rev 1, lane 11: *B. abortus* (REF 544), lane 12: positive control, *B. abortus* S 19, lane 13: negative control with sterile water.

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Supporting Information
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