FULL PAPER *Bacteriology*

Epidemiology of Bovine Brucellosis by a Combination of Rose Bengal Test and Indirect ELISA in the Five Districts of Uganda

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ABSTRACT. A serological survey on bovine brucellosis was carried out 3 times between 2007 and 2009 in 3 districts (Kiboga, Mpigi and Kiruhura) in western Uganda and 2 (Kumi and Mbale) in the east employing the rose bengal test (RBT) for infected-herd screening and an indirect ELISA (iELISA) for testing the serostatus of individual animals. The animal prevalence was significantly higher in the 3 districts of the west (mean 21.5% in 2009) compared with the 2 districts (mean 3.4% in 2008) in the east ($P<0.0001$), though a significant difference was not observed between Kumi and Mpigi in 2008. In the west, it was the lowest in Mpigi, but a significant increase was observed between 2008 (5.3%) and 2009 (30.0%), as in Kiruhura, in which the prevalence increased from 8.1% in 2007 to 16.8% in 2009. A similar trend was also observed in Kumi, namely, the seropositivity significantly increased from 2.3% in 2007 to 6.2% in 2008 and became remarkably higher than in Mbale (0.64%). As a result, the farm prevalence was also higher in the west, especially in Kiboga in 2007 (77.8%) and 2008 (65.6%), and Mpigi in 2009 (70.8%). The linear predictor of the fitted generalized linear model proved that the logit of RBT positivity increased linearly over the increase in percent positivity values. This study demonstrated an example of an unaided self-help survey as one of the control measures in Uganda.

KEY WORDS: brucellosis, cattle, epidemiology, prevalence survey, Uganda.

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Brucellosis is a major zoonotic disease of public health importance in domestic animals, wild animals and humans [15]. It is one of the neglected zoonotic diseases given little attention and often persists in the poorest and most vulnerable populations [5]. The disease occurs worldwide, and it has often been misdiagnosed as malaria in human cases in Africa, which causes a serious threat to public health. The major source of infection in people is undoubtedly domestic animals, and therefore, disease control in these animals is the most important measure to reduce human cases.

Several studies have reported that the *Brucella* seroprevalence in domestic animals in Uganda was generally high and varied depending on the area; however, the results appear to be inconsistent [1, 8, 9, 14]. In order to illuminate a reliable clear picture of the disease status quo in cattle, the major domestic animal in Uganda, 3 districts in the west and 2 in the east were selected as study areas, since the rearing system in the west is different from the one in the east and these 2 systems represent the majority of the cattle farming styles in Uganda. The diagnostic tools employed in this study were prepared or at least can be prepared domestically for sustainable control of animal brucellosis, because most attempts at animal disease control in Uganda have heavily relied on international donors or foreign research funding and ended when only halfway completed due to budget or time limitations. Accordingly, one of the purposes of the study was to investigate the possibility of unaided self-help control measures.

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MATERIALS AND METHODS

Study districts: Sampling took place in 3 districts (Kiboga, Mpigi and Kiruhura) in western Uganda and 2 districts (Kumi and Mbale) in eastern Uganda (Fig. 1). The districts occupy 4045.5, 3601.9, 4608, 2848 and 559.8 km^2 of Uganda (241,000 km^2), respectively. In the former 3 districts, the land is hilly and has a mean altitude of 1200 m above sea level. The climate is equatorial temperate with mean minimum and maximum annual temperatures of 15 and 26°C, respectively. There are 2 rainy seasons (March-May and September-December), and the annual rainfall is over 1,000 mm [6]. Dairy farming is the prevailing industry and mainly uses a local breed, the Ankole, which is often cross-bred with the Holstein-Friesian. The farm sizes are relatively large, and farms occasionally have hundreds of animals. On the other hand, in the latter 2 districts, the topography is generally quite flat, with a mean altitude of 1,000 m. The climate is a subtropical type with more distinctive rainy

and dry seasons, and consequently, it is hotter and has less rainfall than the west, especially in Kumi [6]. The majority of the farmers in this region have a small number of cattle and goats as a kind of asset, and large-scale farming is not common. The major cattle breed in the east is the Boran zebu or East African Shorthorn zebu.

Samples: Three samplings were planned, i.e., in 2007 (October and November), 2008 (August and September) and 2009 (February and March). Although 3 samplings were carried out in Kiboga and Mbale, only 2 samplings were actually implemented in the remaining 3 districts, with sampling not being performed in 2007 in Mpigi, in 2008 in Kiruhura and in 2009 in Kumi. In Kiboga, Mpigi and Kiruhura, randomly selected farms were visited for sampling, while animals were brought by farmers to the sampling sites for sample collection in Kumi and Mbale, as the farms in the east were considerably small. Animals vaccinated against brucellosis were excluded from sampling. The number of farms or areas visited and the number of animals sampled are summarized in Table 1 along with the sampling times.

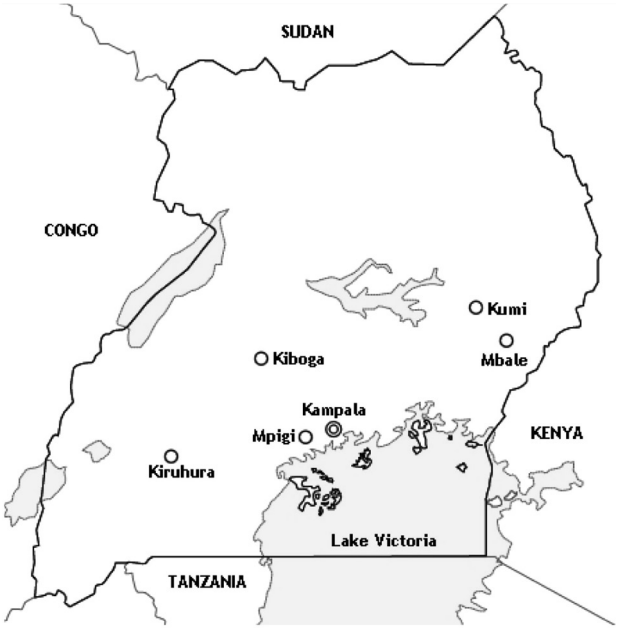

Fig. 1. A map of Uganda showing the principal towns of the five study districts and Kampala, the capital city

All the blood samples were brought to the basic laboratories in the respective district veterinary offices, processed for serum separation and examined by the rose bengal test (RBT) for screening of the positive farms (infected herds). All the sera from the positive farms were brought to the National Animal Disease Diagnostic and Epidemiology Centre in Entebbe and further tested by an indirect ELISA (iELISA) for confirmation. One hundred and four (104) samples of the 388 collected in the 5 sub-counties of Kumi in 2007 contained plasma with ethylenediaminetetraacetic acid (EDTA) and were found to be unsuitable for the RBT, as EDTA causes nonspecific coagulation with the RB reagent. Therefore, only 284 samples were tested by the RBT, and all 388 samples were examined by the iELISA.

Rose bengal test: The reagent for the RBT was prepared as described by the OIE [15] using *Brucella abortus* strain 99, provided by National Institute of Animal Health (NIAH), Thailand, and tested by comparing the reactivity with a commercial product, BENGATEST, Synbiotics Corp., utilizing 200 sera sampled in Kiboga in 2007.

Indirect ELISA: All the reagents including control sera (strongly positive, C++; weakly positive, C+; negative, C-) except for the conjugate (rec-Protein G-peroxidase conjugate, catalog No. 10-1223, Zymed), were provided by the NIAH, and the antigen was prepared according to the method of the OIE [15]. The test procedure and validation of the results were the same as described by Ekgatat *et al.* [2]. The optical density values were read using an ELISA reader (Multiskan MS, Labsystems), and then percent positivities

able 1. The results of serological surveys of bovine brucellosis by a combination of the RBT and iELISA in 5 districts of Uganda

Sampling Time	District	Farms or areas visited[a)]	Samples collected	RBT positive	iELISA positive	Positive farms or areas[a)]
2007 Oct.–Nov.	Kumi	9/7[b)]	388	5	9 (2.3)[c)]	4/9 (44.4)
	Mbale	11/4	549	2	0 (0)	0/11 (0)
	Kiboga	9/5	237	63	46 (19.4)	7/9 (77.8)
	Kiruhura	20/5	418	66	34 (8.1)	10/20 (50)
2008 Aug. –Sep.	Kumi	12/11	566	18	35 (6.2)	5/12 (41.7)
	Mbale	11/4	467	1	3 (0.64)	2/12 (18.2)
	Kiboga	32/12	560	66	89 (15.9)	21/32 (65.6)
	Mpigi	19/6	377	20	20 (5.3)	7/19 (36.8)
2009 Feb. –Mar.	Mbale	8/3	494	1	3 (0.61)	2/8 (25)
	Kiboga	42/8	497	32	88 (17.7)	11/42 (26.2)
	Mpigi	24/4	340	39	103 (30)	17/24 (70.8)
	Kiruhura	45/7	400	77	67 (16.8)	25/45 (55.6)

a) Area signifies a group of herds gathered at a sampling site in eastern Uganda.
b) The number of farms or areas sampled / the number of sub-counties visited.
c) The numbers in parentheses indicate percentages (i.e., prevalence).

(PPs) were calculated as follows:

$$PP(\%) = \frac{replicate\ OD\ value\ of\ \ each\ control\ or\ sample}{median\ OD\ value\ of\ \ C\ +\ +\ control} \times 100$$

The cut-off value was set to 40% for cattle sera as suggested by Ekgatat *et al*. [2–4]. The seroprevalence was calculated based on the number of iELISA-positive samples out of the number of samples collected in the respective district. The overall positive-animal rates in the study districts (the averages of the 3 districts in the west in 2009 and of the 2 in the east in 2008) were also provided for better understanding of the prevalence tendency in the country.

Statistical analysis: For comparison of the *Brucella* prevalence in cattle between the different districts for the same sampling time, and the different sampling times in the same district, the chi-square test was employed using a 2 × 2 contingency table and Fisher's exact probabilities were calculated. The differences in the antibody levels between any two of the districts or the different years in the same district were analyzed by the Wilcoxon rank-sum test.

In addition, in order to analyze the relationship between the RBT results and the PP values, a generalized linear model (GLM) was fitted to a part of the data consisting of 1,365 sera sampled in Kiboga, Mpigi and Kiruhura in 2008 and 2009 using R, version 2.13.0 (www.stats.bris.ac.uk/R). The random distribution was defined as binomial, and the link was logit, thus specifying logistic modeling. The response variable was status of the RBT (coded "1" for positive and "0" for negative), and the explanatory covariate was the PP of the iELISA as a continuous value. The kappa value was also calculated in order to evaluate the consistency between the RBT and iELISA results.

RESULTS

Validation of the tests: The reactivity of the homemade RB reagent was tested utilizing 100 cattle and 100 goat sera sampled on 4 farms (25 each for both species) in Kiboga in 2007. Both (homemade and commercial) reagents showed identical results, 31 positive samples for cattle and 17 positive samples for goats. The validation of the iELISA depended on the PP values of the 3 positive controls. When one or more of these values fell either above or below the diagnostic threshold PP values set by Ekgatat *et al.* [3], the results derived from the particular plate were rejected, and all the samples applied onto the plate were retested. Occasionally faulty plates were experienced.

For the correlation between the RBT and iELISA, the linear predictor of the fitted GLM was $\eta_i = -4.11 + 0.0588 x_{i,PP}$, and thus, the probabilities of RBT positivity $_{\eta_i}$ were calculated using the inverse link function $E[Y_i] = \frac{e^{\eta_i}}{1+e^{\eta_i}}$ where Y_i (i=1, ..., 1365) was the response under the explanatory covariates $x_{i,PP}$ (in%). The P-values for both the intercept and slope parameter of the covariates (PP) were smaller than 0.0001, and the P-value for the fitness of this logit model was 1, which proved that the logit of RBT positivity increased linearly over the increase in PP values. The probability values for RBT being positive were 14.8% for the PP value of 40, 85.5% for PP 100 and 99.1% for PP 150. There was an increasing probability of RBT-positive result with increasing PP values. In addition, the calculated kappa value was 0.5155, which indicated that the RBT results were moderately consistent with the iELISA results.

Brucella seroprevalence in the study districts: The seroprevalence revealed in this study is summarized in Table 1. The overall positive-animal rates in the study districts were 21.5% in the west in 2009 and 3.4% in the east in 2008. The individual animal prevalence was significantly higher in the 3 districts of the west compared with the 2 districts in the east (P<0.01), though a significant difference was not observed between Kumi and Mpigi in 2008. Within the 3 districts of the west, it was the lowest in Mpigi, but a significant increase (P<0.01) was observed between 2008 (5.3%) and 2009 (30.0%) as in Kiruhura (P<0.01), in which the prevalence increased from 8.1% in 2007 to 16.8% in 2009. A similar trend was also observed in Kumi (P<0.01), namely, the seropositivity significantly increased from 2.3% in 2007 to 6.2% in 2008 and became remarkably higher than in Mbale (0.64%). As a result, the farm prevalence was also higher in the west. In Kiboga, the prevalence was 77.8% in 2007 and 65.6% in 2008 and then declined to 26.2% in 2009. On the other hand, in Mpigi, it increased from 36.8% in 2008 to 70.8% in 2009. In the east, the area prevalence was higher in Kumi (about 40%) than in Mbale (about 20%), although a significant difference was not demonstrated as only a few areas were visited for sampling.

Antibody levels of cattle in Kiboga and Mpigi: The antibody levels in cattle in Kiboga and Mpigi were analyzed based on the PP values (Table 2), since a number of RBT-negative samples turned out to be positive by the iELISA (Table 1). In both of these districts, the antibody levels of all the cattle at the RBT-positive farms were lower in 2008 than in 2009. On the other hand, those of the iELISA-positive cattle at the same farms were unchanged in Kiboga, while those in 2009 were significantly lower than in 2008 in Mpigi.

DISCUSSION

Validity of the diagnostic tools employed: Both the RBT and iELISA are prescribed tests for international trade, and the details of the antigen production and test procedures are well described by the OIE [15]. The RBT was regarded as adequate as a screening test for detecting infected herds and to guarantee the absence of infection in brucellosis-free herds; therefore, it was employed in the same manner in this study. The RB reagent prepared in the study was tested compared with a commercial product as mentioned earlier, and it was confirmed that the reactivity was as good as that of the commercial product in 200 samples from infected herds in Kiboga.

The iELISA employed in this study was developed according to the method of the OIE [15] by a group at National Institute of Animal Health, Thailand, headed by Dr. Egkatat, who kindly provided all the reagents and control sera for the study. Its sensitivity, specificity and accuracy were proven to

Table 2. The antibody levels of the cattle from the RBT-positive farms in Kiboga and Mpigi in 2008 and 2009

ELISA percent positivity (PP)	Kiboga		Mpigi	
	2008	2009	2008	2009
0≤PP<10	4 (0.9)[a)]	3 (1.5)	9 (5.4)	0
10≤PP<20	115 (27.3)	30 (14.6)	68 (40.7)	20 (6.9)
20≤PP<30	153 (36.2)	51 (25)	65 (38.9)	70 (24.3)
30≤PP<40	52 (12.3)	32 (15.6)	7 (4.2)	71 (24.7)
40≤PP<50	29 (6.9)	23 (11.3)	5 (3.0)	49 (17.0)
50≤PP<60	15 (3.6)	20 (9.8)	4 (2.4)	38 (13.2)
60≤PP<70	8 (1.9)	15 (7.4)	0	14 (4.9)
70≤PP<80	7 (1.7)	11 (5.4)	1 (0.6)	7 (2.4)
80≤PP<90	6 (1.4)	3 (1.5)	1 (0.6)	4 (1.4)
90≤PP<100	6 (1.4)	2 (1.0)	0	2 (0.7)
100≤PP	27 (6.4)	14 (6.9)	7 (4.2)	13 (4.5)
Total count	422 (100)	204 (100)	167 (100)	288 (100)
PP mean (all)[b)]	35.6	45.2	27.2	43.2
PP S.D. (all)[b)]	29.7	32.9	29.1	24.5
Wilcoxon test[b, c)]	0.0000001		0.0000000	
Positive count	98 (23.2)	88 (43.1)	18 (10.8)	127 (44.1)
PP mean (+)[d)]	78.2	72.2	92.3	61.9
PP S.D. (+)[d)]	36.2	34.0	54.2	26.2
Wilcoxon test[c, d)]	0.584		0.0465	

a) The numbers in parentheses indicate percentages.
b) The statistics for all the samples tested by the iELISA, i.e., samples from the positive farms.
c) The Wilcoxon rank-sum test.
d) The statistics for the iELISA-positive samples, i.e., the samples with PPs of over 40.

be 99.4, 99.9 and 99.9% for samples from cattle and 99.2, 99.9 and 99.9% for samples from goats respectively utilizing the isolation of *B. abortus* or *B. melitensis* as a gold standard in testing of 5,617 samples from cattle (317 infected and 5,300 noninfected) and 1,272 samples from goats (82 positives and 1,190 negatives) by Ekgatat *et al*. [2–4]. The system was further validated in testing of 9,877 sera from the field by employing the complement fixation test as a standard. The system utilizes 3 control sera (strongly positive, weakly positive and negative) for internal quality control, and the PP cut-off value was determined to be 40% for samples from cattle using the frequency distribution and receiver-operator characteristic curve.

Gómez *et al*. [7] evaluated seven tests for diagnosis of human brucellosis and demonstrated that the sensitivity of the ELISA was not higher than that of the conventional tests (RBT, microagglutination test, microtiter-adapted Coombs test and immunocapture-agglutination test: Brucellacapt). On the other hand, Mainar-Jaime *et al*. [10] reported that serial use of pairs of specificity-correlated serological tests (RBT, complement fixation and competitive ELISA) resulted in specificities lower than expected when brucellosis false-positive serological reactions occur in cattle and concluded that highly specific tests, such as the iELISA used alone, may be more adequate than serial testing. When the iELISA was compared with the competitive ELISA, both showed very high specificities (99–100%) for bovine, ovine and caprine samples from noninfected animals [17]. According to the further study by Pajuaba *et al*. [16], the iELISA using *B. abortus* smooth lipopolysaccharide antigen and protein A-HPRO conjugate for preferential detection of the IgG2 subclass was shown to be suitable for serological distinction between S-19-vaccinated and S-19-infected cows, which indicates that replacement of the protein G-HPRO conjugate employed in this study by protein A conjugate may improve the system so as to differentiate naturally infected cattle from vaccinated ones.

The fitted GLM demonstrated that the RBT positivity would increase as the PP values increased. The *P*-value for the GLM fitted to our data was 1, which suggests that the fit of the model was satisfactory. The *P*-value for the slope parameter of PP was smaller than 0.01, which indicates an effective covariate. However, the fitted GLM demonstrated that only 14.8% of the samples with a PP value of 40% (cut-off titer for cattle samples) produced an RBT-positive result, which means the iELISA is more likely to produce positive results than the RBT when infected herds are tested, and thus, a number of infected animals will be misdiagnosed when tested only by the RBT. Actually, this sort of case was observed in Kiboga and Mpigi in 2009, and the details are discussed in the latter part of this report. If the model is fitted without the data from Kiboga and Mpigi in 2009, the probabilities are expected to be higher. Furthermore, the model can be improved, if more covariates are included such as age, abortion history or even other complementary diagnosis results.

In summary, a combination of the RBT for screening infected herds and the iELISA for identifying infected individuals was considered to be a quite appropriate and effective diagnostic tool for large-scale serological survey of

Table 3. The seroprevalence of bovine brucellosis in the sub-counties of Kumi District

Sub-county	2007			2008		
	Areas	Number	Positives	Areas	Number	Positives
Town Council	1	3	1 (33.3)[a)]	1	31	1 (3.2)[a)]
Kumi	−	−	−	1	46	0
Atutur	−	−	−	1	23	0
Kanyumu		−	−	1	66	0
Ongino	1	63	2 (3.2)	1	71	6 (8.5)
Mukongolo	1	38	0	1	87	8 (9.2)
Nyero	−	−	−	1	51	0
Ngora	2	62	1 (1.6)	1	35	0
Kobwin	2	78	0	1	34	0
Kapir	1	72	5 (6.9)	2	84	19 (21.4)
Mukura	1	72	0	1	38	0
Total	9	388	9 (2.3)	12	566	35 (6.2)

a) The numbers in parentheses indicate percentages.

brucellosis. In addition to the RBT reagent, we have already initiated the antigen production for the iELISA, which will enable us to continue the study nationwide and hopefully contribute to the control of brucellosis in Uganda.

Seroprevalences in the study districts: Several studies have reported the seroprevalence of brucellosis in Uganda. Bernard *et al*. [1] carried out an intensive survey of bovine brucellosis and tuberculosis in the Mbarara milk basin, which included the present Kiruhura District, and determined a herd prevalence of 55.6% (n=315) and individual animal prevalence of 15.8% (n=10,562) by the RBT. These results are very close to the prevalences in Kiruhura in 2007, which were 50% (=10/20, herd prevalence) and 15.8% (=66/418, animal prevalence by the RBT). However, the animal prevalence by the iELISA was 6.2% and increased significantly to 16.8% in 2009 (Table 1). A retrospective study was undertaken by Mwebe *et al*. [14] that obtained data from the 3 major veterinary laboratories in the country and reported a seroprevalence of 7% (n=694) in Kumi, 44% (n=9) in Mbale, 0% (n=524) in Kiboga, 45% in Mpigi (n=143) and 20% (n=138) in Kiruhura. Although the data were recorded between the period of 1998 and 2008, prevalence rates reported are widely different from those found in this study except for Kumi and Kiruhura (Table 1). The numbers of samples reported by Mwebe *et al*. [14] were rather small, and the high incidences in Mbale and Mpigi as well as the low incidence in Kiboga are hardly credible. Their finding that the trend of the disease has declined over years also contradicts our results. The present study proved that the disease has been spreading in Kumi, Mpigi and Kiruhura, and the detailed prevalence revealed particular high-risk areas in the districts such as Kapir Sub-county in Kumi (Table 3). Kapir is a kind of place in which animals from the surrounding districts mingle, which is considered to result in increase of the incidence.

The risk factors of the infection are also discussed in several studies. Magona *et al*. [9] reported that the risk of natural *B. abortus* infection was higher under the pastoral system than the zero-grazing system and among older cattle. Our surveys elucidated that the seroprevalence was significantly higher in the west than in the east. In all the sampling areas in the present study, animals are kept basically under the pastoral system but freely graze within a large farm in the west, while they are usually constrained with rope or fences in the east. In addition, the biggest difference between the 2 regions is the size of the farms. For example, the average number of cattle per farm in Kumi in 2007 was 3.5 (n=94, 95% CI: 2.7–4.3), while in Kiboga it was 376 (n=9, 95% CI: 213–539). As a result, the large farm size and free grazing appear to be the most important risk factors based on our study. Makita *et al*. [11] also reported that large herd size and history of abortion were identified as risk factors at the herd level in the Kampala economic zone, Uganda.

Most cases of human brucellosis have resulted from consumption of raw milk transported from peri-urban and rural areas of Kampala and/or dairy production areas outside Kampala [12, 13], which includes Kiboga, Mpigi and Kiruhura Districts. Therefore, it is very important to control brucellosis in these districts for public health, as 12.6% of informally marketed milk in urban Kampala was estimated to be contaminated with *B. abortus* at purchase, causing an annual incidence rate of 5.8 per 10,000 people [12].

Antibody levels in Kiboga and Mpigi: In 2009, an excessive number of iELISA-positive samples was found in Kiboga and Mpigi (Table 1). When the antibody titers represented as PP were examined, those in 2008 were significantly lower than those in 2009 in Kiboga. This is thought to have been caused by the fact that the proportion of the iELISA-positives samples from the positive farms was nearly twice as large in 2009, while the antibody levels of the iELISA-positive samples showed no difference between the 2 sampling occasions (Table 2). However, the RBT-positive samples (n=32, PP=99.1 ± 41.8) showed significantly higher PP values than the iELISA-positive samples (P<0.01 by the Wilcoxon rank-sum test), which indicates that a number of the latter samples had rather low PP values and accordingly explains the excessive number of iELISA-positive samples compared with RBT-positive samples. In the case of Mpigi, the PP values in 2008 were significantly lower than those in 2009, but the iELISA-positive samples in 2009 demonstrat-

ed significantly lower PPs than those in 2008, which clearly caused the outcome of three times as many iELISA-positives samples than RBT-positive samples. These findings imply that the infection was just about in the expansion phase at the third sampling time in the 2 districts and that the proportion of RBT-positive animals is expected to increase since the antibody levels will rise as the infection develops. In addition, the RBT-negative herds may also harbor iELISA-positive animals, and ideally, all the samples should probably be tested by the iELISA for us to get an accurate picture of the conditions of the disease.

Consequently, close surveillance should be carried out regularly in the 3 districts of the west and also in Kumi for the control of brucellosis in domestic animals, as the prevalence is likely to increase drastically in the near future. Finding a control strategy in Kiboga (the farm prevalence decreased from 77.8% to 26.2%) as well as a risk factor (s) in Mpigi (increased from 36.8% to 70.8%) will certainly be beneficial for us to combat the disease.

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