Brucella sero-prevalence and modifiable risk factors among predisposed cattle keepers and consumers of un-pasteurized milk in Mbarara and Kampala districts, Uganda

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Abstract:

Background: Brucellosis is an important zoonotic disease in developing countries yet it is often not recognized, goes unreported and does not attract public health action by these governments including Uganda.

Objective: To estimate the sero-prevalence and assess modifiable risk factors associated with Brucella seropositivity in cattle keepers and consumers of unpasteurized milk in Uganda.

Methods: One group comprised of 161 individuals randomly selected from households living on farms that had Brucella sero-positive cattle and/or goats in Mbarara District from an earlier survey; the second group comprised of 168 randomly selected individuals attending an HIV voluntary counseling and testing clinic in Kampala District. Sera samples were tested using Rapid Plate Agglutination Test, Standard Tube Agglutination Test and cELISA.

Results: The sero-prevalence of brucellosis among exposed cattle keepers in Mbarara and consumers of unpasteurised milk in Kampala Districts was 5.8% (95%CI: 3.3%, 8.3%) and 9% (95%CI: 13.3%, 4.7%), respectively. Consumption of unboiled milk was significantly (p=0.004) associated with seropositivity in Mbarara District. There was no association between sero- positivity with age, sex and awareness of human brucellosis.

Conclusion: Human brucellosis is prevalent among livestock rearing communities and consumers of unpasteurised milk. The continued consumption of unboiled milk is a major health risk.

Key words: Brucellosis, Modifiable risk factors, Sero-prevalence, unpasteurised milk, cELISA

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Introduction

Brucellosis also known as Malta fever or undulant fever for the disease in humans[1] is among the most common and important zoonotic disease globally especially in developing countries yet it often is not recognized, goes unreported and does not attract public health action by these governments[2, 3].

There are six known species with numerous biotypes. Brucella abortus, and B. melitensis cause disease in cattle, pigs sheep and, goats,respectively, resulting in important economic losses. Although B. melitensis is the most pathogenic for humans, Brucella species show cross-species infection particularly with B. melitensis (Corbel et al., 2006)[3].

Humans usually acquire brucellosis through contact with infected animals or consumption of contaminated milk or milk products[4]. Although B. melitensis is the most pathogenic compared to B. abortus for humans, the consumption of goat milk in Uganda is not common[5] (Ndyabahinduka et al, 1978). Brucellosis is also recognized as an occupational hazard for farmers, veterinarians, and workers in the meat industry in areas with enzootic B. abortus and/ or B. melitensis.

A recent study among abattoir workers in Uganda reported a brucella seropositivity of 10% (95%CI: 6–16; n=232)[6] (Nabukenya et al, 2013). Symptoms of acute brucellosis caused by B. abortus and /or B. melitensis are flu-like and are highly non-specific. Chronic brucellosis is an insidious disease with vague symptoms that might be confused with other diseases affecting various organ systems[5,7]. The varied and sometimes deceptive manifestation of localized, sub-acute or chronic infections may lead to miss-diagnosis or delayed diagnosis if the attending clinician has a low index of suspicion. The disease is a zoonosis of worldwide distribution and a common cause of economic loss and ill health among animals and human populations. Although the incidence of brucellosis has decreased significantly in developed countries[6,8], the disease remains a major public health threat in many developing countries including Uganda[7, 8, 9,10].

In Uganda, the disease in animals remains a private matter with good with control measures either-measures either lacking or difficult to implement. Brucellosis cases in the human population largely go un-noticed probably because the disease is not among those routinely screened for in health centers in Uganda. Consequently there is poor knowledge, if any concerning the prevalence and epidemiology of this disease in the human population in Uganda. To date there is no comprehensive study to high light the status of human brucellosis in Uganda. This study sought to establish the sero-prevalence of Brucella antibodies among exposed cattle keepers in Mbarara district where brucellosis is known to be endemic among livestock,[9, 10 11,12] among consumers of un-pasteurized milk in Kampala district, and also to identify modifiable risk factors for the disease.

Methods

Sampling and sample size determination

The study population was comprised of two groups. One group consisted of individuals from farms where cattle and/or goats tested positive for Brucella from an earlier survey in Mbarara district (south western Uganda – where 98 herds of cattle and goats from three agro-ecological zones were studied - unpublished) and the second group included individuals recruited at HIV counseling and testing clinic in Kampala who answered in affirmative for consuming unpasteurized milk. For the Mbarara sample, three teams each comprising of two medical laboratory technicians under the supervision of a medical doctor visited households where cattle and/or goats tested positive for Brucella in a previous study. For the Kampala sample participants were asked to disclose their laboratory identification numbers for purposes of accessing aliquots of their blood sample used in HIV screening, participants were also interviewed on their feeding habits and lifestyles.

Assuming Brucella sero-prevalence of 11.9%[11,13] and 18%[8,10] for Mbarara and Kampala districts respectively, with a 95% confidence that the error in the estimate will not exceed 5% and using standard survey formula[12,14], a total of 329 individuals were studied; 161 from Mbarara and 168 from Kampala. At least seven participants were sampled from each of the 26 households that had cattle and goats that tested positive for Brucella. Blood was centrifuged and serum stored at -200C until it was tested using three tests – the buffered/Rose Bengal plate agglutination test (RPAT), the standard tube agglutination test (STAT) and the competitive ELISA (cELISA) test at Makerere University's then Faculty of Veterinary Medicine (now the College of Veterinary Medicine, Animal Resources and Biosecurity, COVAB.

Ethical Issues

The rights of the human participants were clearly explained to each one of them. All participants signed consent forms written in their own local language which guaranteed that all the information and samples collected were to be used only for the intended purpose, and their identify would remain confidential. Ethical approval for the study was sought and granted by the Research and Ethics Committee of Mbarara University of Science and Technology (MUST) Medical School and the Uganda National Council of Science and Technology (UNCST). The field team included qualified and registered medical laboratory technicians under the supervision of two medical doctors who were charged with collecting serum samples from the human subjects.

Data handling and analysis

Serological Assay

All samples were tested by both the buffered plate agglutination test (BPAT) using febrile antigens and competitive enzyme linked immunosorbent assay (cELISA). Samples that tested positive on the BPAT were re-tested on the serum tube agglutination test (STAT) for confirmation. The febrile antigens multi-screening kits together with the positive and negative human control sera used in the agglutination tests were supplied by Human Gesellschaft fur Biochemia und Diagnostica mbH, Germany. The cELISA kits were supplied by Veterinary Laboratories Agency (VLA) of the Department for Environment, Food and Rural Affairs, UK.

Buffered Plate Agglutination Test (BPAT)

The test was carried out as described by Lucero and Bolpe[13,15]. Briefly the test antigen together with the test and control sera were removed from the refrigerator

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and allowed to thaw for about 30 minutes then mixed thoroughly but gently. Equal volumes of 50 ul for both the test serum and the antigen were placed side by side on separate cells of the white slide. The antigen and test sera were then mixed with separate disposable sticks and the fluid spread over the entire area of a particular cell. The slide was then rocked gently back and forth for up to 1 minute. The results were read under bright artificial light. Any sample with visible agglutination was designated positive.

Standard/Serum Tube Agglutination Test (STAT)

The test was performed as previously described by Lucero and Bolpe[13,15]. Briefly, all test reagents and serum samples were thawed to room temperature and then mixed thoroughly but gently prior to use. Then 20 ul of each serum sample were diluted with 1.98 ul of NaCL (9g/l); six double dilutions were then made for each serum sample and the controls from 1/20 through 1/640. One drop of the antigen was then added to each of the tubes. The tubes were closed, contents mixed thoroughly and incubated at 37^0C for 24 hours. The sample was categorized as positive if there was a coarse, compact agglutination with clearing of the supernatant.

The results were negative if the supernatant was unchanged in its appearance and showed a swirl when the tube was flicked. The highest dilution to give agglutination was recorded as the titer for that sample. However, any sample that showed agglutination at the dilution of 1/20 and above was designated positive.

Competitive Enzyme Linked Assay (cELISA)

The test was performed as described by Nielsen and others[14,16]. Pre-coated 96 wells of microtiter plates with B. melitensis LPS antigen (Veterinary Laboratory Agency, UK) were used. Briefly 20 ul of each test serum in duplicate was dispensed per well, leaving the last two columns for the controls (serum and conjugate controls). Immediately 100 ul of the conjugate (Goat anti-mouse immunoglobulin G antibody conjugated to horseradish peroxidase diluted in phosphate buffer solution) were dispensed into each well. After vigorously shaking for about 2 minutes on an automatic shaker, the plate was incubated for 30 minutes at room temperature on a rotary shaker. The plate was then washed 5 times with Tween 20 and Na_2HPO_4 solution.

Finally 100 ul of the substrate (Urea hydrogen peroxide) and chromogen (OPD) mixture were added to each well and the plate left for 10–15 minutes at room temperature. The reaction was then stopped with 100 ul of citric acid solution and the OD (Optic Density) of the plate read with a microtiter plate reader at 450 nm. Lack of colour development indicated a positive sample, as indicated by the colorless wells. A positive/negative cut off was calculated as 60% of the mean of the optical density (OD) of the 4 conjugate control wells. Any sample that gave an OD value equal to or below this cut off value was regarded as positive.

Data collection and analysis

A standard structured questionnaire was administered by personal interview to the sampled study individuals. This comprised data on host attributes like age, sex, religion and place of residence. Data on milk feeding habits and contact with livestock were also collected. Raw data was entered, validated and stored in Microsoft Access (MS Office 2003, Microsoft®). Validated data was then exported to Statistical Package for Social Science (SPSS 12.0 for Windows) for analysis. All the postulated risk factors were first assessed for significance and their association with the disease outcome (cELISA test status of a farm) by computing their respective odds ratios and chi square values before offeringfeeding them into a generalized linear mixed model (GLMM) 15 and executed in SAS IML macro (SAS institute Inc., version 6, 1985)[15,17] to further study the relationship between the postulated risk factors and the disease outcome for purposes of identifying modifiable ones. Significance at initial screening and for the final model was set at $p<0.25$ and $p<0.05$, respectively.

Results

Descriptive statistics

The majority (62.6%, n=329) of the study group were male; this proportional difference was reflected in the two District, Kampala and Mbarara. The Kampala population was older than that of Mbarara with average of 31 years and 29 years (Median 29 years vs. 23years), respectively.

Overall, the majority (69.3%, n=329) of people interviewed took milk at least once every day with those in Mbarara District taking significantly ($p<0.05$) more milk than Kampala (85.5%, 95%CI 90.9,80.1 vs 53.6%, 95%CI 61.1,46.1). Consumption of un-boiled milk remained a common practice especially in Mbarara than in Kampala Districts (37.9% vs 16.7%) but was not significantly deifferent. The prevalence of brucellosis-like symptoms (prolonged fevers not responding to antimalarial treatment) among the individuals interviewed was high at 81.2% (n=325). This contrasted with the poor knowledge of human brucellosis among the people, where 69.6% (n=329) of all the people interviewed had never heard of the disease (Table 1)

Table 1: Distribution of hypothesized risk factors by District

	No	78.6(132)	60.2(97)	69.6(229)

Variable	Levels	Kampala %(n=168)	Mbarara %(n=161)	Overall %(n=325)
Sex	Female	38.7(65)	36(58)	37.4(123)
	Male	61.3(103)	64(103)	62.6(206)
Milk consumption frequency	At least once/day	53.6(90)	85.7(138)	69.3(228)
	Less than once/day	46.3(78)	14.3(23)	30.7(101)
Consumption of un-boiled milk	Yes	16.7(28)	37.9(55)	26.5(83)
	No	83.3(140)	62(90)	73.5(230)
Contact with Animals	Yes	17.3(29)	93.2(150)	54.4(179)
	No	82.7(139)	6.8(11)	45.6(150)
History of brucellosis-like symptoms 12months preceding study	Yes	88.7(149)	71.4(115)	81.2(264)
	No	8.9(15)	28.6(46)	18.8(61)
Human brucellosis awareness	Yes	21.4(36)	39.8(64)	30.4(100)

*For Mbarara, n=145

** For Kampala n=164; Overall total (n=325)

Individual Brucella sero-prevalence

The overall sero-prevalence on screening with BPAT at individual level was 15.2% (n=329 samples) of which 19 (5.8%, n=329) were confirmed positive at a STAT titer of 1:20 and above. However, cELISA test found 16 (4.9%, n=329) samples positive (Table 2). The proportion of individuals with Brucella antibodies on all the three tests (BPAT, STAT and cELISA) were higher in Mbarara than in Kampala Districts (Table 2).

Table 2:Individual level sero-prevalence based on BPAT, STAT, cELISA and STAT+cELISA by District.

Area	No sampled	Seroprevalence %±SE(p)			
		RBT	STAT	cELISA	STAT/cELISA
Mbarara	161	18.0 ± 0.03	7.5± 0.02	9.3± 0.02	13.0±0.03
Kampala	168	12.5± 0.03	4.2 ± 0.02	0.6 ± 0.005	4.8 ± 0.02
Total	329	15.2 ± 0.02	5.8 ± 0.01	4.9 ± 0.01	8.8± 0.02

Although 19 of 329 samples (5.8%) gave positive results with STAT titers of 1/20, Table 2 shows that although sero-positivity at different dilutions appeared higher in Mbarara compared to Kampala Districts, significant difference was shown with cELISA only. In addition, there was a significant ($p<0.05$) difference in the number of people with titers >1/160 in Mbarara than in Kampala samples (Table 3).

Table 3: Distribution of STAT titers in the study area.

Area	No sampled (n)	STAT titers (%, 95%CI) 1/20	≥1/40	≥1/160
Mbarara	161	7.5 (8.3, 3.4)	3.7 (6.6, 0.8)	2.5 (4.9, 0.1)
Kampala	168	4.2 (7.2, 1.2)	1.8 (3.8, 0.0)	0.0 (0, 0)
Total	329	5.8 (8.3, 3.3)	2.7 (4.4, 1.0)	1.2 (2.4, 0.0)

When individuals were re-classified according to reacting positively on both STAT and cELISA tests, in an attempt to improve sensitivity, 8.8% (29/329) were sero-positive. Sero-prevalence differed between the Mbarara and Kampala groups (Table 2).

Rrisk factors associated with Brucella sero-positivity.

In order to improve the sensitivity of the two tests (STAT and cELISA), individuals were re classified as being positive if one gave a positive result on any of the two tests i.e. interpretation in parallel. Univariate univariable analysis showed that the consumption of unboiled milk was significantly (p=0.004) associated with seropositivity in Mbarara District. No significant difference was found between the age, sexes and human brucellosis awareness both in Mbarara and Kampala Districts (p >0.05) (Table 4).

Table 4: Distribution of sero-positivity by hypothesized risk factors and district.

Variable/District	Frequency	Seropositive cases (%) STAT/cELISA	p – value
Sex			
Mbarara			
Female	58	10.3 (6)	p= 0.44
Male	103	14.6(15)	
Kampala			
Female	65	7.7(5)	p= 0.26
Male	103	2.9(3)	
Age			
Mbarara			
<29yrs	99	10.0(10)	p= 0.30
≥29yrs	62	17.7(7)	
Kampala			
<31yrs	100	6.0(6)	p= 0.15
≥31yrs	68	2.9(2)	
Consumption of unboiled milk *			
Mbarara			
Yes	55	23.6(13)	p= 0.004*
No	90	7.8(7)	
Kampala			
Yes	28	3.6(1)	p= 0.99
No	140	5.0(7)	
Knowledge of human brucellosis			
Mbarara			
Yes	64	10.9(7)	p= 0.52
No	97	14.4(14)	
Kampala			
Yes	36	2.7(1)	p= 0.99
No	132	5.3(7)	

* For Mbarara n=145

Discussion

This study has shown that overall, the STAT sero-prevalence of human brucellosis among cattle keepers in Mbarara and consumers of milk in Kampala was 5.8%. This observation agrees closely with other studies – an earlier study, one in a big hospital in Kampala among febrile patients and another among abattoir workers in Kampala and Mabrara that reported sero-prevalences of 13% and 10%, respectively,[9]. Three percent (4/161) of the STAT positive people in Mbarara District had high antibody titers (>1/160), which was indicative of active infection. These four people with high STAT titers were also experiencing brucellosis-like clinical signs suggesting acute brucellosis infection. Although no single test provides 100% specificity and sensitivity, STAT remains the test of choice in diagnosis. In the presence of appropriate signs and symptoms, a presumptive diagnosis of brucellosis is usually defined serologically as a standard tube agglutination titer of 1:160 or greater[16,18]. This is however time-consuming, be it in sero-epidemiological studies,where a large number of sera samples have to be processed or in hospital/medical laboratories, where treatment of brucellosis patients has to be commenced soon. Therefore, other less laborious and faster turn-around diagnostic tests like competitive enzyme-linked immunoassay (cELISA) are currently used in the diagnosis of human diagnosis[17,19]. cELISA has the advantage of being fairly rapid to perform, somewhat faster than STAT, and cross-reacts less with other antigens (or antibodies) than the conventional tests. In the current study the sero-prevalence obtained by STAT was not different (p<0.05) from that for cELISA.

Data on the sero-prevalence of human brucellosis in developing countries is very limited indeed. Previous studies carried out predominantly in the Mediterranean region have reported sero-prevalence estimates ranging from 8% in Jordan[18,20] to 15% in Saudi Arabia[19,21]. In sub- Saharan Africa sero-prevalence estimates of 5.3% in Nigeria[20,22] and 10%-13.3% in Uganda[7,6,9] have been reported.

Isolation of Brucella microorganisms by blood cultures is confirmatory of brucellosis; however in practice it is difficult because of early tissue localization of the bacteria and the exacting culture requirements. In practice, blood cultures are positive in 10% - 30% of brucellosis cases[16,18], and the remainder is diagnosed serologically. None the less brucellosis diagnosis particularly in endemic areas poses enormous challenges. Past studies have reported a low specificity for the commonly used serological tests (RBT and STAT) in endemic areas and in patients with a long history of brucellosis[17,19], [21,23]. Competitive enzyme immunoassay (cELISA) has high specificity and sensitivity (99.7% and 98.3), and is useful for evaluating treatment effectiveness, for monitoring clinical conditions, and for prognosis[17]. In the present study sera were screened by PBAT, followed by STAT as the confirmatory test.

In addition opportunities to diagnose brucellosis with cELISA were also explored.

In order to improve the sensitivity of the two tests (STAT and cELISA), individuals were reclassified as being positive if one gave a positive result on any of the two tests i.e. interpretation in parallel. Using this criterion, 29 out of 329 sera samples (9%) were positive. Basing on this classification consumption of unboiled milk was significantly (p= 0.004) associated with sero-positivity in Mbarara District. This is in agreement with other studies[22,24]. In Kampala District unlike Mbarara, there was no significant (p=0.99) difference in Brucella seropositivity between consumers of unboiled raw milk and those who do not. This may be that freshly drawn milk which the consumers in Mbarara District commonly take is more infective compared their Kampala counterparts who are most likely to consume adulterated milk. Moreover the study has shown that the proportion of people who consume larger quantities of milk per day, (i.e > 500mL/day), is higher in Mbarara than in Kampala Districts (85% vs. 28%).

Conclusion

This study has clearly demonstrated that human brucellosis is still prevalent and that consumption of unboiled raw milk continues to be practiced despite the risk it poses to human health due to brucellosis. This study found no significant association (p>0.05) with age and sex and brucella sero-status. This calls for immediate and deliberate efforts by the authorities to institute prevention and control measures. The most effective way to control the disease in man is by elimination of the infected animals, and vaccination of the health ones in order to reduce the risk of those in regular contact with animals, and to produce brucellosis free animal products. Avoiding consumption of raw milk and proper heat treatment of milk is important for effective prevention of the disease in humans. However local customs like those encouraging consumption of freshly drawn milk (locally called amakamo in Mbarara district) is a challenge and may greatly hinder wide application of such measures. Consequently health education should always be an integral part of every phase of disease prevention and control. Close cooperation and joint supervision between the ministries of Health and Agriculture, Animal Industries and Fisheries should be encouraged.

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