This study identified and characterized *Brucella* species in the informal milk chain in Uganda. A total of 324 cattle bulk milk samples were screened for the genus *Brucella* by real-time PCR with primers targeting the *bcsp31* gene and further characterized by the *omp25* gene. Of the samples tested, 6.5% were positive for *Brucella* species. In the *omp25* phylogeny, the study sequences were found to form a separate clade within the branch containing *B. abortus* sequences. The study shows that informally marketed cattle milk in Uganda is a likely risk factor for human brucellosis and confirms that *B. abortus* is present in the cattle population. This information is important for potential future control measures, such as vaccination of cattle.

**Keywords:** Africa; brucellosis; bulk milk; milk delivery chain; PCR; *bcsp31*; *omp25*

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and 2012, 324 bovine bulk milk samples were collected from the two districts, see Rock et al. (12). In brief, the samples were collected directly from the containers of informal milk sellers and milk deliverers at the roadside, at milk-collecting centers, and at boiling points. Ethical clearance was obtained as described in Rock et al. (12).

**Bacterial reference strains**

DNA from the vaccine strains *B. melitensis* Rev. 1, *B. abortus* RB51, and *B. suis* in the commercial InGene Bruce-ladder V kit (Ingenasa, Madrid, Spain) was used as positive controls in all *Brucella* PCR-assays. In the 16S rRNA real-time PCR assay, the positive controls consisted of DNA from the bacterial strains *Pseudomonas aeruginosa* B683 and *Treponema T2378*.

**Genomic DNA extraction and real-time PCR detection**

Genomic DNA was extracted using a phase separation technique with phenol:chloroform:isoamyl alcohol (Sigma-Aldrich, St. Louis, MO, USA), recommended by the Animal Health and Veterinary Laboratories Agency *Brucella* research division in Weybridge, UK. The quantities and purities of the extracted DNA from all samples were determined by optical density measurement using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). DNA extracts were stored at −20°C and were analyzed in 2014 for the genus *Brucella* as described by Probert et al. (13) with the modification that the assay was run as a singleplex with primers and probe targeting the bscp31 gene. Extracts were randomly selected for analysis in order to prevent cross-contamination during handling. Two negative controls consisting of DEPC water (Invitrogen, Thermo Fisher Scientific, Stockholm, Sweden) were included in each run to detect PCR contamination. The limit of detection was determined to a DNA concentration of 3.6 ng µL⁻¹ extract. Samples with a cycle threshold (Ct) value of ≤40 were interpreted as positive.

**Molecular typing of bulk milk samples by omp25**

*Brucella* spp. were characterized in five strong positive extracts, four from Gulu and one from Soroti, using the *omp25* gene (13). The limited number of samples characterized was due to limited amount of DNA extracts. The expected size of the *omp25* amplicons was 523 base pairs (bp). Weak positive samples in the bscp31 assay gave weak band in conventional PCR and were not enough for sequencing.

**DNA sequencing and sequence analyses**

Purified PCR products were sent to Macrogen Europe (Amsterdam, the Netherlands) for Sanger sequencing. Purification was performed with ExoSAP-IT (Affymetrix, USB, Santa Clara, CA, USA), according to the manufacturer’s instructions. Sequencing primers for the *omp25* amplicons were the same as for PCR.

Sequences were edited and assembled with the CLC Main Workbench 7 (CLC Bio-Qiagen, Aarhus, Denmark). Contigs and individual sequences were blasted (www.ncbi.nlm.nih.gov/BLAST/) and aligned in the MEGA6 software, using the MUSCLE algorithm. The total length of the alignment, excluding non-overlapping sequences, was 455 nucleotides. Phylogenetic trees were generated in MEGA6 and *Ochrobactrum anthropi* was used as outgroup to root the trees (14). The corresponding sequence of the *O. anthropi* *omp25* gene was identified from the whole genome entry of *O. anthropi* (Accession number CP000758). Phylogenetic relationships were inferred using the neighbor-joining (NJ) and maximum-likelihood (ML) algorithms. Bootstrapping of the NJ method data based on 1,000 replicates assessed the resulting tree topology. All sequences in this study have been deposited in GenBank under the accession numbers KY038989-KY038992.

**Results**

**Detection of Brucella spp. DNA in cattle bulk milk by real-time PCR**

To investigate the presence of inhibitors and the effectiveness of the extraction, all samples were analyzed by a 16S real-time PCR. Bacterial DNA was present in the majority of the DNA extracts with similar fluorescence signal, indicating extraction success.

It was shown that *Brucella* spp. DNA was present in 6.5% (21/324; 95% confidence interval (CI) 3.8–9.2) of informally marketed raw cattle milk collected from street sellers and milk deliverers in two districts in Uganda. Of those were 5.3% (10/188; 95% CI 2.1–8.5) from Gulu and 8.1% (11/136; 95% CI 3.5–12.7) from Soroti.

**Characterization of Brucella spp. in cattle bulk milk**

Four (three from Gulu and one from Soroti) out of five extracts were successfully sequenced to ascertain *Brucella* spp. identification. Comparison of *omp25* sequences revealed only single nucleotide substitutions and indicated a similarity of more than 98% between study and reference sequences of closely related *Brucella* spp. Study sequences were 100% identical to each other and exhibited the highest level of sequence similarity to *B. abortus* (99.6% nucleotide similarity, 453/455 bp).

Phylogenetic analysis of *omp25* sequences indicated that *B. abortus* was present in the marketed raw cattle milk from Gulu and Soroti Districts. The sequences from the cattle bulk milk were found to form a separate clade within the branch containing *B. abortus* sequences. The NJ and ML trees exhibited similar topology and were in
overall agreement with current Brucella taxonomy – separating three out of the four closely related classical Brucella spp. into separate sub-branches; B. melitensis, B. abortus, and B. canis. A B. abortus–B. melitensis clade appeared in both analyses.

Discussion

This study showed that B. abortus is the probable species found in bulk milk aimed for human consumption and complements previous findings by Mugizi et al. (9) and Rock et al. (12). Identifying infection species is important if control measures, such as vaccination, are to be implemented in the future. This study also indicates that informally marketed raw cattle milk is a probable risk factor for human brucellosis if consumed raw, even if presence of Brucella DNA does not give information of the presence of viable Brucella bacteria. The study also showed that proportionally more samples from Soroti contained Brucella DNA compared with samples from Gulu. The same relation with respect to antibodies against Brucella was shown by Rock et al. (12).

The real-time PCR results indicated low Brucella bacterial DNA load in the bulk milk extracts, which was expected since few Brucella bacteria in general are excreted in cattle milk (4) and pooling of milk from different sources might have a dilution effect. A greater analytical sensitivity has been observed when using the insertion element IS711 as target, in real-time PCR assays for detection of Brucella at the genus level (15). In this study, IS711 was not targeted due to limited amount of extracts.

All Brucella spp. have a genome similarity of more than 90%. Effective genetic markers for distinguishing closely related Brucella spp. and their biovars from each other are therefore difficult to identify. In this study, the Brucella genus–specific omp25 gene was used as a genetic marker. The similarity of the omp25 sequences proved to be more than 98%, clearly indicating that the sequences belong to the genus Brucella, and further confirming the presence of Brucella spp. in the omp25 phylogeny. Study sequences were found to group with B. abortus sequences. Additionally, a close relationship was found between B. abortus and B. melitensis. This agrees with other analyses across multiple gene markers (16, 17).

This study confirms previous findings that Brucella abortus is the species infecting cattle in Uganda. This information is important if control options at the production level in the milk chain, such as vaccination of cattle against B. abortus, would be discussed.

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References

1. Havelaar AH, Kirk MD, Torgerson PR, Gibb HJ, Hald T, Lake RJ, et al. World Health Organization global estimates and regional comparisons of the burden of foodborne disease in 2010. PLoS Med 2015; 12: e1001923.
2. Food and Agriculture Organization of the United Nations (2011). World Livestock 2011 – Livestock in food security. Rome: FAO.
3. Whatmore AM, Davison N, Cloackaert A, Al Dahouk S, Zygmunt MS, Brew SD, et al. Brucella papionis sp. nov., isolated from baboons (Papio spp.). Int J Syst Evol Microbiol 2014; 64: 4120–8.
4. The World Organization for Animal Health (2009). OIE Terrestrial Manual 2009. Chapter 2.4.3. Bovine brucellosis. OIE; Available from: http://web.oie.int/eng/normes/MMANUAL/A_Index.htm [cited 19 November 2014].
5. Al Dahouk S, Sprague LD, Neubauer H. New developments in the diagnostic procedures for zoonotic brucellosis in humans. Rev Sci Tech 2013; 32: 177–88.
6. Corbel M, Leberg S, Cosivi O. Brucellosis in humans and animals. Geneva: WHO Press; 2006.
7. Godfroid J, Scholz HC, Barbier T, Nicolas C, Wattiau P, Fretin D, et al. Brucellosis at the animal/ecosystem/human interface at the beginning of the 21st century. Prev Vet Med 2011; 102: 118–31.
8. Makita K, Fevre EM, Waiswa C, Eislter MC, Welburn SC. How human brucellosis incidence in urban Kampala can be reduced most efficiently? A stochastic risk assessment of informally-marketed milk. PLoS One 2010; 5: e14188.
9. Mugizi DR, Muradrassoli S, Boquist S, Erume J, Nasinyama GW, Waiswa C, et al. Isolation and molecular characterization of Brucella isolates in cattle milk in Uganda. Biomed Res Int 2015; 2015: 720413.
10. Mugizi DR, Boqvist S, Nasinyama GW, Waiswa C, Ikwap K, Rock K, et al. Prevalence of and factors associated with Brucella sero-positivity in cattle in urban and peri-urban Gulu and Soroti towns of Uganda. J Vet Med Sci 2015; 77: 557–64.
11. Miller R, Nakavuma JL, Ssaajjakambwe P, Vudriko P, Musisi N, Kaneene JB. The prevalence of brucellosis in cattle, goats and humans in rural Uganda: a comparative study. Transbound Emerg Dis 2015; 63: e197–210. doi: http://dx.doi.org/10.1111/tbed.12332
12. Rock KT, Mugizi DR, Stahl K, Magnusson U, Boquist S. The milk delivery chain and presence of Brucella spp. antibodies in bulk milk in Uganda. Trop Anim Health Prod 2016; 48: 985–94.
13. Probert WS, Schrader KN, Khuong NY, Bystrum SL, Graves MH. Real-time multiplex PCR assay for detection of Brucella spp., B. abortus, and B. melitensis. J Clin Microbiol 2004; 42: 1290–3.
14. Velasco J, Romero C, López-Goñ i l, Leiva J, Díaz R, Moriyón I. Evaluation of the relatedness of Brucella spp. and Ochrobactrum anthropi and description of Ochrobactrum intermedium sp. nov., a new species with a closer relationship to Brucella spp. Int J Syst Bacteriol 1998; 48: 759–68.

15. Bounaadja L, Albert D, Chenais B, Henault S, Zygmunt MS, Poliak S, et al. Real-time PCR for identification of Brucella spp: a comparative study of IS711, bcsP31 and per target genes. Vet Microbiol 2009; 137: 156–64.

16. Halling SM, Peterson-Burch BD, Bricker BJ, Zuerner RL, Qing Z, Li LL, et al. Completion of the genome sequence of Brucella abortus and comparison to the highly similar genomes of Brucella melitensis and Brucella suis. J Bacteriol 2005; 187: 2715–26.

17. Whatmore AM, Perrett LL, MacMillan AP. Characterisation of the genetic diversity of Brucella by multilocus sequencing. BMC Microbiol 2007; 7: 34.