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SHORT COMMUNICATION

Molecular detection and characterization of *Brucella* species in raw informally marketed milk from Uganda

Tove Hoffman, MMSc[1,2], Kim Rock, DVM[3], Denis Rwabiita Mugizi, PhD[4], Shaman Muradrasoli, PhD[1,3,5], Elisabeth Lindahl-Rajala, DVM[6], Joseph Erume, PhD, DVM[4], Ulf Magnusson, DVM, Professor[6], Åke Lundkvist, PhD, Professor[1,2] and Sofia Boqvist, DVM, Associate Professor[3]*

[1]Zoonosis Science Center, Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden; [2]Department of Medical Science, Uppsala University, Uppsala, Sweden; [3]Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden; [4]College of Veterinary Medicine, Animal Resources and Bio-Security, Makerere University, Kampala, Uganda; [5]Division of Clinical Microbiology, Department of Laboratory Medicine, Karolinska University Hospital, Stockholm, Sweden; [6]Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden

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*

*Correspondence to: Sofia Boqvist, Department of Biomedical Sciences and Veterinary Public Health, Swedish University of Agricultural Sciences, Uppsala, Sweden, Email: Sofia.Boqvist@slu.se

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Brucellosis is a widespread but neglected bacterial zoonosis of global importance (1). Besides being a public health threat, brucellosis also has a serious global economic impact as it causes severe production losses due to infertility, abortions, and reduced milk production in goats, sheep, cattle, and swine (2).

The bacterial genus *Brucella* consists of 11 species, of which some have been further subtyped into biotypes or biovars (3). The genomes of *Brucella* species (spp.) have a similarity at the nucleotide level exceeding 90% (4). *Brucella melitensis*, *B. abortus*, some *B. suis* biovars and *B. canis* are most commonly reported in domestic animals and are also known to be zoonotic (5). Even if *Brucella* exhibits host species preference, cross-infections to other animal species may occur (6). The major modes of transmission of *Brucella* infection to humans are through contact with infected animals, fetal membranes, aborted fetuses, and consumption of unpasteurized dairy products (7).

Cattle brucellosis is endemic in Uganda and human brucellosis is an important disease in the country (8). It has been shown that the *Brucella* seroprevalence at dairy herd level ranged between 28 and 44% (9–11) and that antibodies against *Brucella* spp. were present in 11 and 40% of samples of bulk milk (12). It has also been shown that *B. abortus* is infecting dairy cattle (9), but there is a lack of knowledge about presence of *Brucella* in the informal milk delivery chain in Uganda, here defined as small-scale dairy farmers selling fresh milk without uniform processing (12).

The aim of this study was to identify and characterize *Brucella* spp. in the final step of the informal milk delivery chain in Uganda, through molecular techniques.

Materials and methods

Study design and sample collection

The Gulu and Soroti Districts were included as they are two rapidly growing urban areas located in Northern and Eastern Uganda. Small-scale livestock holding is an important source of livelihood in these areas. In 2011

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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 *bcsp31* 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 *bcsp31* 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

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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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Conflict of interest and funding

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