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Molecular detection of *Campylobacter* species from human and cattle faecal samples in Kilosa district, Tanzania

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Abstract

Background: A growing number of *Campylobacter* species other than *C. jejuni* and *C. coli* have been considered as emerging human and animal pathogens. However, the contribution of these species to human gastroenteritis is poorly documented. This study aimed at detecting *Campylobacter* species from human and cattle faecal samples in Kilosa district, Tanzania using Polymerase Chain Reaction (PCR) amplification of the 16S rRNA gene, and Sanger sequencing.

Methods: A total number of 100 faecal samples (70 from human and 30 from cattle) were collected from diarrheic and non-diarrheic patients and healthy cattle in Kilosa district, Tanzania from July to October 2019. Species identification was conducted by PCR and 16S rRNA sequencing. The phylogenetic analysis was carried out by comparison of the 16S rRNA gene sequences to reference strains by the Neighbor-Joining method in MEGA X.

Results: *Campylobacter* species detection rate by PCR was 65.7% (46/70) and 20% (6/30) in humans and cattle, respectively. There were five human diarrheic cases, four showed *Campylobacter* presence and two were from children ≤ 15 years of age. In humans, the 16S rRNA sequencing revealed that *C. concisus* was the most predominant species occurring at a frequency of 37.8% (14/37), followed by uncultured *Campylobacter* spp. 24.3% (9/37) and *C. hominis* 21.6% (8/37). The least represented species were *C. jejuni* and *C. lanienae* all occurring at 2.7% (1/37). In cattle, five (100%) sequenced PCR products matched with *C. lanienae*. Phylogenetic analysis revealed that *Campylobacter* 16S rRNA sequences were closely related to *C. concisus*, uncultured *Campylobacter* sp., *C. hominis*, and *C. gracilis*.

Conclusion: The non-*C. jejuni*/*C. coli* species are present in human and cattle faecal samples and their true occurrence is probably under-reported due to shortcomings of conventional techniques used in most diagnostic microbiology laboratories. Based on our findings, we recommend that molecular techniques be adopted for direct detection of *Campylobacter* species during routine laboratory screening and surveillance studies.

Keywords: *Campylobacter*, molecular diagnostics, polymerase chain reaction, sequencing, gastroenteritis, Tanzania

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Introduction

Campylobacter, one of the zoonotic pathogens causing gastroenteritis, is responsible for 96 million cases of diarrhoea each year [1, 2]. *Campylobacter* has also been reported to cause infertility in cattle and abortions in sheep, goats, and cattle [3]. The incidence of human cases of campylobacteriosis has been increasing in many countries throughout the world [4, 5]. In Africa, the prevalence varies from 7.7–18.5%, and *Campylobacter* is persistently found in stools of both diarrheic and non-diarrheic children [7, 8, 9], often associated with poor hygiene and sanitation [9].

Over time, human gastroenteritis has been linked to *C. jejuni* and *C. coli* accounting for more than 95% of *Campylobacter* isolations [11, 12]. However, atypical *Campylobacter* species are gaining considerable attention as important human and animal pathogens [13–15]. Infections caused by *Campylobacter* are usually under-reported due to difficulties in isolation procedures [16, 17]. For instance, it has been estimated that 40% of the bacteria from human faeces diagnosed through microscopy cannot be cultured in the laboratory [17]. In contrast to other gastrointestinal pathogens, the culturing of *Campylobacter* species is laborious due to their microaerophilic nature and vulnerability to temperature fluctuations [18]. Furthermore, commonly used selective media and added antimicrobials may inhibit the growth of certain *Campylobacter* species [19]. Moreover, *Campylobacter* species may become dormant as viable but non-culturable (VBNC) forms difficult to grow on commonly used media [20]. Consequently, the epidemiology and role of non-*C. jejuni*/*C. coli* species to human gastroenteritis are not fully understood [11, 22].

The emerging *Campylobacter* species have been neglected but the integration of molecular techniques and suitable culture media in current diagnostic tests has helped in promoting the awareness of atypical species as relevant human and animal pathogens [5, 22]. *Campylobacter concisus* has been associated with gastroenteritis, brain abscess, Crohn's disease, arthritis, and ulcerative colitis [14, 23–24]. *Campylobacter hominis* has been isolated in a blood sample of a septicemic patient [24], while *C. gracilis* has been associated with bacteremia, head infections, periodontitis, and empyema [23, 26]. In cattle, the most commonly reported species are *C. fetus*, *C. lanienae*, *C. sputorum*, *C. jejuni*, and *C. hyointestinalis* [28–30].

The polymerase chain reaction (PCR) and other molecular diagnostic tests based on nucleic acids are attractive due to their benefits including their higher sensitivity, ease-of-use, improved turnaround time, relatively low cost, and potential to be fully automated [32–34]. The breakthrough in technology and easy access to commercial kits has led to shifting from traditional laboratory diagnostic techniques to newer molecular ones [32]. The analysis of the 16S rRNA gene by PCR and sequencing techniques has assisted in the phylogenetic identification of *Campylobacter* species including those unidentified by conventional techniques [27, 35]. Nevertheless, *Campylobacter* isolation by culture is still useful as it allows the isolation of pure colonies and testing of antimicrobial susceptibilities [34].

In Tanzania and most of the low and middle-income countries (LMICs), the reports on the role of *Campylobacter* spp. in gastroenteritis are scanty due to limited capacity in laboratory diagnosis and absence of national surveillance programs [7, 37]. Thus, the information available for both human and animal campylobacteriosis is limited [36] which undermines its importance as a public health concern. The current study aimed at molecular detection of *Campylobacter* species from

human and cattle faecal samples in Kilosa district, Tanzania using PCR amplification of the 16S rRNA gene and Sanger sequencing.

Materials and methods

Study design and sample collection

This cross-sectional study was conducted in Kilosa District of central Tanzania (6° S and 8°S, and between 36° 30' and 38°E) from July 2019 to October 2019. Human stool samples were randomly obtained from patients with abdominal discomfort seeking medical care at Kilosa District Hospital during the time of the study. Cattle faecal samples were randomly collected from healthy lactating cows using sterile gloves. A total of 70 human stool samples and 30 cattle rectal grab faecal samples were collected in sterile dry screw-top containers containing Dimethyl Sulfoxide (DMSO), packed in a cool box and transported to the Sokoine University of Agriculture Microbiology laboratory for DNA extraction within 8 hours of collection.

DNA extraction and *Campylobacter* species identification

Approximately, 1g faecal sample in DMSO was diluted (10% wt/vol) in buffered peptone water (BPW) (9 ml) and vortexed until the sample was thoroughly homogenized. Then, 200 µL of the homogenized faecal sample was used for genomic DNA extraction using Quick-DNA™ Faecal/Soil Microbe Microprep Kit (Zymo Research Corp, Irvine, CA, USA) based on the manufacturer's instructions. Eluted DNA concentration and purity were checked using a NanoDrop™ spectrophotometer (Biochrom, Cambridge, England) before storage at -20°C.

Detection of *Campylobacter* was done by multiplex PCR, using *C. jejuni* *cj0414* gene primers (C1; C3) and *C. coli* *ask* gene primers (CC18F; CC519R) as previously described [37]. Then, PCR of the 16S rRNA gene was performed on DNA samples negative for *C. jejuni* and *C. coli* using genus-specific primers including a 19bp-forward primer (C412F) and an 18bp-reverse primer with complementary sequence (C1228R) as previously described [38]. Positive control DNA was extracted from *Campylobacter jejuni* (ATCC® 33560™); while deionized water was included as the negative control. The PCR final volume was 25 µL, including 12.5 µL of 2X Master Mix (Thermo Fisher Scientific, Seoul, South Korea), 1 µL (10 µM) of C412F primer, 1 µL (10 µM) of C1228R primer, 1 µL of template DNA, and 9.5 µL of sterile deionized water. All primers were made by Integrated DNA Technologies, Inc. (Singapore Science Park, Singapore).

The DNA amplification was performed using the model MiniAmp™ Plus Thermal Cycler (Applied Biosystems, MA, USA). The cycling conditions used were one cycle of 95°C for 5 minutes, 35 cycles each of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. The PCR products were held at 4°C before analysis. PCR products (5µl) were mixed with 2µl Loading STAR (Dyne bio, Seongnam-si, Korea) diluted with 5µl of nuclease-free water and analyzed by gel electrophoresis: 10µl of the mixture was loaded onto 1.5% SeaKem® LE Agarose gel (Lonza Inc.-Rockland, ME, USA) in 0.5X TAE buffer. After electrophoresis, PCR product bands were visualized using a Dual UV Transilluminator (Core Bio System, Huntington Beach, CA, USA) under ultraviolet (UV) light and photographed with iBright™ CL1000 Imaging System (Thermo Fisher Scientific, Seoul, South Korea). The size of the amplification products (816bp) obtained was compared to the Dyne 100 bp DNA ladder (Dyne bio, Seongnam-si, Korea). The PCR products (816 bp) were purified using Pure Link™ Quick

PCR purification Kit (Invitrogen, Vilnius, Lithuania) and sequenced at SolGent (Solutions for Genetic technologies, Daejeon, South Korea) using the *Campylobacter* genus-specific primers by Sanger method.

Data analysis

The data were analyzed with GraphPad Prism 8.4.0 (GraphPad Software, La Jolla, CA, USA). Descriptive statistics (frequencies and percentage) were computed to determine proportions for different attributes. The GenBank sequences with the best, and the high scoring matches with sequences of this study were selected using the NCBI BLASTN search. Sequences were edited, aligned, and analyzed using BioEdit sequence alignment software (version 7.2.6.1) [40, 41]. Multiple sequence alignment by Muscle [41], computation of evolutionary distances by the Jukes-Cantor method [42], and the phylogenetic tree building by the Neighbor-Joining (NJ) method [43], were done in MEGA X software (MEGA Inc, Englewood, NJ) [44]. The phylogenetic analysis was carried out by comparing the sequences of this study to 16S rRNA genes of reference strains downloaded from LPSN (<https://lpsn.dsmz.de>). To confirm the reliability of our analysis, bootstrap analysis was performed with 1,000 resampled datasets and it was taken to represent the evolutionary history of the taxa analyzed [45]. All the 16S rRNA gene sequences derived from sequencing were submitted to GenBank for obtaining accession numbers.

Results

A total of 70 (male=35; female=35) human stool samples were collected. The age of patients ranged from 2 to 89 years with 14.3% being children ≤ 15 years of age. Overall, the detection rate of *Campylobacter* spp. in human samples was 65.7%. The PCR products with predicted size (816bp) were obtained in some of the screened samples (Figure 1). Of the *Campylobacter* spp. positive samples (n=46), 24 (52.2%) were from females and 22 (47.8%) were from males. *Campylobacter* species were detected in nine of the 10 (90%) children ≤ 15 years old. There were five diarrheic cases, of which four showed *Campylobacter* presence. Of the diarrheic patients, two were children ≤ 15 years. In cattle, all the 30 samples were collected from lactating cows with age varying between 3.4-8 years. Of the 30 samples, six (20%) exhibited *Campylobacter* spp.

Figure 1: PCR products using the 16S rRNA primers specific for *Campylobacter* genus. Lanes: 1: 100bp molecular weight marker; 2-4: bands from human samples; 5: negative control; 6: positive control; 7-8: bands from cattle samples (Attachment).

The results of sequencing confirmed the presence of *Campylobacter* species in all submitted sequences (37 from humans and 5 from cattle). The remaining PCR products did not give enough quantity of DNA (required by the sequencing company) after the purification step. The species were confirmed based on percent identity (above 99%), the query cover, and the E-value. In humans, *C. concisus* was the most prevalent (37.8%), followed by uncultured *Campylobacter* spp. (24.3%), and *C. hominis* (21.6%). *Campylobacter lanienae*, and *C. jejuni* occurred at a frequency of 2.7% each (Figure 2). For cattle, all the five (100%) 16S rRNA sequences matched with *C. lanienae*.

Figure 2: Distribution of *Campylobacter* species from human samples identified by sequencing (Attachment).

The 16S rRNA genes of *Campylobacter* spp. from this study were compared with 16S rRNA sequences of different strains of *Campylobacter* spp. by BLASTN search. Following submission to the GenBank, gene sequences were allocated with the following accession numbers: MT126449 to MT126453; MT130973 to MT130991; and MT131150 to MT131167.

The phylogenetic analysis was carried out by comparing the 16S rRNA genes of this study to 16S rRNA genes of reference strains (*C. concisus*, *C. hominis*, *C. gracilis*, and *C. lanienae*) and uncultured *Campylobacter*. The analysis of sequence data from *Campylobacter* species of this study revealed a high nucleotide sequence similarity to different reference strains. *Campylobacter hominis* clustered closer to *C. gracilis* than it was with *C. concisus*. It was also noted that *C. lanienae* formed a separate cluster at the bottom of the tree. Uncultured *Campylobacter* and *Campylobacter* spp. RM 12175 were also found among the sequences of this study (Figure 3). The tree was rooted using *H. aurati* and *A. molluscorum*.

Figure 3: Molecular phylogenetic analysis of *Campylobacter* species using 16S rRNA sequences by the neighbor-joining method. Bootstrap values (%) based on 1000 replicates are indicated at nodes. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X. Reference strains were included in the analysis. The tree was rooted using *H. aurati* and *A. molluscorum* (Attachment).

Discussion

For many years *C. jejuni* and *C. coli* have been the most commonly reported species associated with gastroenteritis in humans [11]. Nonetheless, advances in molecular diagnostic techniques proved that isolation by culture contributes to increased detection rates of *C. jejuni* and *C. coli* over other species which bias both the outcome of the diagnosis and the contribution of other species to *Campylobacter* infections [46]. The routine culture-based method fails to detect over a third of *Campylobacter* positive samples. Bullman et al. [46] attribute the scarcity of atypical *Campylobacter* species reported so far to the limitations and bias of culture-based methods. Therefore, this study reports the detection of *Campylobacter* spp. in both humans and cattle by molecular methods. To the best of our knowledge, this is the first report in Tanzania detecting the presence of *Campylobacter* species directly from feces without a culture-based approach. Previous authors adopted culture methods [49–51] which could not give a true picture of *Campylobacter*-related infections as some could have failed to grow on selective media.

The reported detection rate of *Campylobacter* spp. (65.7%) in humans was similar to that reported in Nigeria [50] but higher than those reported previously in Tanzania [53, 54], Fiji [53], India [54], and Cambodia [9]. The difference could be attributed to the sampling strategy, PCR conditions, and geographically related variations. *Campylobacter concisus* and *C. hominis* were the most predominant species occurring at 37.8% and 21.6%, respectively. This concurs with the findings reported in Denmark [55] and Australia [56]. Lastovica et al. [16] reported that *C. concisus* was the second species with a higher prevalence after *C. jejuni*. However, *C. concisus* has been isolated

from diarrheic patients without other pathogenic microorganisms suggesting it to be an emerging cause of human gastroenteritis [16, 57].

Campylobacter lanienae was detected in both cattle and humans. It has been previously recovered from healthy livestock [57]. This *Campylobacter* spp. has been reported as a probable aetiology of human gastroenteritis [58]. However, it is suggested that *C. lanienae* has restricted pathogenicity or be a non-pathogenic *Campylobacter* [22]. The observed frequency of occurrence of the species in this study was higher than the one previously reported by Inglis & Kalischuk [28]. Further characterization of *C. lanienae* could shed more light on its genetic diversity and source [57].

The data on the concurrent isolation of *Campylobacter* species in both humans and cattle are limited. In this study, the detection rates were 65.7% and 20%, in humans and cattle, respectively. Our findings showed higher detection rates when compared to the rates reported earlier in Tanzania [59], and Cambodia [9]. Further comparative studies on *Campylobacter* species isolated from humans and cattle are necessary to understand their epidemiology and be able to conclude on source attribution.

The phylogenetic analysis based on the 16S rRNA gene is of paramount importance for bacterial taxonomy [60] and it has been applied to *Brucella* [61] and *Campylobacter* identification [5, 61]. Our findings concur with the reported species of non-*C. jejuni*/ *C. coli* group with *C. concisus* being the predominant species [63]. In cattle, our results are in agreement with previously reported occurrence where *C. lanienae* had higher proportions compared to *C. jejuni* and *C. hyointestinalis* [57].

The current study had some limitations including the sample size and lack of culture-based species identification. The sample size used could not allow us to estimate the prevalence or generalize the findings at national or regional levels. Considering that we did not culture the stool samples, the comparison is made based on previous studies carried in sometimes different conditions or settings. However, this study highlights the advantages of molecular methods over the culture-based one.

Conclusions

The findings of this study highlight the higher prevalence of less frequently isolated *Campylobacter* species (*C. concisus* and *C. hominis*) in patients with gastroenteritis. *Campylobacter lanienae* was detected in both human and cattle faecal samples. These *Campylobacter* species are often neglected due to their cultural behavior and fastidious nature but have proven to be zoonotic with a public health concern. It is therefore important that health practitioners and public health authorities recognize the possibilities of occurrence of *Campylobacter* species other than *C. jejuni* and *C. coli* which are not tested on a routine basis in many countries and go unreported. Molecular-based techniques offer an alternative to culture-based methods especially when it comes to the atypical *Campylobacter* species as they provide results in a short time and up to species level.

List of abbreviations

PCR: polymerase chain reaction; NJ: Neighbor-Joining method; MEGA: Molecular Evolutionary Genetics Analysis; LMICs: low and middle-income countries; 16S rRNA: 16S ribosomal ribonucleic acid, where S stands for Svedberg.

Declarations

Ethics approval and consent to participate

This study was approved by the Medical Research Coordinating Committee of the National Institute for Medical Research in Tanzania (Ref. No.: NIMR/HQ/R.8a/Vol. IX/3070). Stool samples from patients were collected after obtaining informed written consent of the patient or his/her parent/guardian. Farmers consented for sampling their cattle.

Consent for publication

Not applicable

Availability of data and materials

Data generated during this study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable

Authors' contributions

NG carried out the experiments, compiled the results, and wrote the manuscript. KYY and HAM participated in carrying out the experiments. EVGK, MIM, LEGM, RGA, DM, CHP, and KHC participated in the design and helped to revise the manuscript. All authors approved the final version of the manuscript.

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Figures

Figure 1

PCR products using the 16S rRNA primers specific for *Campylobacter* genus. Lanes: 1: 100bp molecular weight marker; 2-4: bands from human samples; 5: negative control; 6: positive control; 7-8: bands from cattle samples.

Figure 2

Distribution of Campylobacter species from human samples identified by sequencing.

Figure 3

Molecular phylogenetic analysis of *Campylobacter* species using 16S rRNA sequences by the neighbor-joining method. Bootstrap values (%) based on 1000 replicates are indicated at nodes. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA X. Reference strains were included in the analysis. The tree was rooted using *H. aurati* and *A. molluscorum*.