Molecular-Based Cross-Species Evaluation of Bovine Coronavirus Infection in Cattle, Sheep and Goats in Ghana.

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Abstract

Background: Apart from the huge worldwide economic losses often occasioned by bovine coronavirus (BCoV) to the livestock industry particularly cattle, continuous surveillance of the virus in cattle and small ruminants is essential in monitoring variations in the virus that could enhance host switching.

Methods: In this study, we collected rectal swabs from a total of 1,498 cattle, sheep and goats. BCoV detection was based on reverse transcriptase polymerase chain reaction. Sanger sequencing of the partial RNA-dependent RNA polymerase (RdRp) region for postive samples were done and nucleotide sequences were compared with homologous sequences from the GenBank.

Results: The study reports a BCoV prevalence of 0.3% consisting of 4 positive cases; 3 goats and 1 cattle. Bayesian majority-rule cladogram comparing partial and full length BCoV RNA-dependent RNA polymerase (RdRp) genes obtained in the study to data from the GenBank revealed that the sequences obtained from this study formed one large monophyletic group with those from different species and countries. The goat sequences were similar to each other and clustered within the same clade. No major variations were thus observed with our isolates and those from elsewhere.

Conclusion: Given that Ghana predominantly practice the extensive and semi-intensive systems of animal rearing, our study highlights the potential for spillover of BCoV to small ruminants in settings with mixed husbandry and limited separation between species. Continuous surveillance of BCoV in the country is thus critical to avert future surge and probable increase in host range.

Background

Bovine Coronavirus (BCoV) belongs to the genus *Betacoronavirus* within the *Coronaviridae* family [1–3]. It is an enveloped, single-stranded and positive-sense RNA virus with a genome size of 32 kb, encoding five main structural proteins: the nucleocapsid, the hemagglutinin esterase, the membrane, the spike (S), and the envelope protein [4].

BCoV has been implicated in severe diarrhea in neonatal calves, winter dysentery in adult cattle and has been associated with respiratory infections in calves and feedlot cattle [5, 6]. Transmission is primarily through respiratory or fecal-oral routes [7], infecting the respiratory (nasal, tracheal, and

3

lung) and intestinal (villi and crypts of the ileum and colon) epithelial cells [8]. Although infection with

BCoV has a low mortality, it generally presents with a high morbidity among cattle of all ages [9].

Outbreaks characteristically occur in autumn and winter, when the virus is most active [10, 11].

Substantial economic toll can be exerted by BCoV infection when a large herd are infected, resulting

in drastic reduction in milk yield [12, 13]

In many developing countries where different animals live in close proximity, there is the possibility of

interspecies transmission of zoonotic diseases. A few studies have reported the detection of BCoV in

small ruminants [14–18].

Despite the health and economic significance of BCoV in livestock, only limited studies have been

conducted to evaluate BCoV infection in livestock in Ghana to date. We have recently reported a high

seroprevalence, cross-species infection and serological determinants of BCoV in cattle, sheep and

goats in Ghana [19]. In this study, we employed a molecular-based detection method to investigate

the presence of BCoVs in rectal samples of cattle, sheep and goats from four major regions in Ghana.

We also characterized, for the first time, the occurrence and the molecular phylogeny of the BCoVs

within the selected regions.

Methods
Study design and area

This was a cross-sectional study conducted between January 2015 to December 2018 in five districts

in four regions of Ghana. Ghana has a tropical climate with an average annual temperature of about

26 °C and the annual rainfall of 736.6 mm/29". Agriculture dominates the economy of Ghana and

extensive farming practices in the country increase the livestock-wildlife-human interface.

Study population, sampling strategy, and data collection

A total of 1,498 animals aged ≥ 6 months, comprising 1,328 cattle, 104 sheep and 66 goats were

included in the study. Sampling was done using a simple two-stage cluster sampling technique. The

Regional Veterinary Officers of the Ministry of Food and Agriculture (MOFA), in the selected regions of

Ghana, were contacted for information on animal populations in their respective regions prior to the

study.

The list provided served as the sampling frame. Prior to the study, a survey was carried out and an

inclusion criteria of animal population (cattle, sheep, and goats) ≥ 1,000 for districts to be eligible for

selection for the study was upheld.

As a result, five (5) districts that fulfilled the criteria were randomly selected. Secondly, farms within

these districts with herd size ≥ 100 animals were also randomly selected. All cattle, sheep, and goats

within the selected farms were included in the study. If a district meets the first criteria, but the

individual farms fail to meet the second criteria, farms which were very close to each other were

pooled together. Sampling was done in the Bongo District in the Upper East; Savelugu and Wale wale

in Northern; Ada West in Greater Accra, and North Tongu in Volta region.

Sample collection, processing and analysis.

Rectal swabs were collected from each animal using sterile swab sticks. The swabs were placed in

pre-labeled cryotubes (SARSTADT, Nümbrecht, Germany) containing a viral RNA stabilization solution,

RNAlater (Applied Biosystems, Foster City, CA, USA). Samples were then transported to the Kumasi

Centre for Collaborative Research (KCCR) for storage at -70 °C prior to laboratory analysis.

Data and sample collection was done on the owners' farms and in their presence, after which animals

were released back to the owners.

Processing of rectal swabs for RNA extraction

Prior to RNA extraction and subsequent laboratory analysis, rectal samples were thawed at room

temperature and vortexed. This was followed by centrifugation at 4000 g for 1–2 min before

aliquoting 140 µl into new sterile tubes.

Testing of Faecal Swabs from Cattle, Sheep, and Goats using RT-PCR

Viral RNA Extraction

Viral RNA was extracted from all 1,498 samples using the spin protocol of the QIAamp Viral RNA Mini

kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. The RNA was eluted in 100 µl

of buffer AVE (pre-warmed at 80^oC). The eluted RNA was stored at -20^oC until they were tested for

BCoV using real-time polymerase chain reaction (RT-PCR).

PCR Product Generation

Detection of bovine CoV RNA was carried out using One-Step RT-PCR Kit (Qiagen, Hilden, Germany)

and HCoV-OC43 primers. The following thermal protocol was used: reverse transcription at 50 °C for

30 min, Taq polymerase inactivation at 95 °C for 15 min, 45 cycles of 95 °C for 15 secs, and 60 °C for 30 secs. Amplification results were acquired at 60 °C. The total volume of the Qiagen one-step Master Mix was 25 µl per sample comprised of RNAse-free water (10.5 µl); Onestep 5 x buffer (5 µl); dNTP (1 µl); Forward primer (1 µl); Reverse primer (1 µl); OC43 probe (0.5 µl); enzyme mix (1 µl) and the 5 µl of the RNA template. The oligonucleotide sequences of the forward and reverse primers used for the amplification were CGATGAGGCTATTCCGACTAGGT and CCTTCCTGAGCCTTCAATATAGTAACC respectively, and the probe sequence used was TCCGCCTGGCACGGTACTCCCT as previously described [20].

Purification of DNA products for sequencing

All bovine coronavirus positive samples were confirmed by means of 1-step reverse transcription-heminested PCR, using primers CoV2A-F (CTTATGGGTTGGGATTATCC) and CoV2A-R (TAATAACAGACAACGCCATCATC) for the first round and the inner primers CoV2A-Rnest a (CCATCATCACTCAGAATCATCA) and CoV2A-Rnest b (CCATCATCAGAAAGAATCATCA) as previously described [21]. This generated a 404-base pair amplicon (Fig. 1) from the RNA-dependent RNA polymerase (RdRp) gene.

The PCR products were then prepared for Sanger sequencing by mixing 5 µL of the product with 2 µL of ExoSAP-IT™ (Thermo Fisher, MA, USA) and incubated at 37°C for 15 minutes. After this, the mixture was incubated at 80°C for 15 minutes and then stored at 4°C until use. A volume of 3 µL of each of these cleaned products was then pipetted into 2 tubes and 6 µL of RNAse-free water added to each tube. A volume of 1 µL of the forward primer was added to one tube and the same volume of nested reverse primers to the other tube to give a 10 µL total volume per tube. Sanger sequencing was done by Seqlab GmbH, GÖttingen Germany.

All obtained sequences from Seqlab were compared to sequences deposited on GenBank via the BLAST Algorithm and were aligned together with reference sequences from the Genbank. A cladogram was constructed using Bayesian inference which compared the partial BCoV RdRp sequence obtained from this study to those from different species and countries.

Statistical Analysis

Categorical data were presented as frequencies (percentages). Analysis of sequence data was done

using the online BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify homologous strains.

Construction of the phylogenetic tree was done by Bayesian inference using MrBayes [22] plugin in

Geneious Prime 2019 (http://www.geneious.com). Graphical presentation was performed using

GraphPad Prism 7 version 7.04 (GraphPad Software, Inc., La Jolla, California USA).

Results

The prevalence of BCoV

The prevalence of BCoV in the entire animal population was 0.3% (4/1.495) (Fig. 2a). Out of the 4

positive cases, 3 were goats whereas 1 was cattle (Fig. 2b). Based on stratification by the sampling

regions, the three BCoV positive goats were from the Upper East region while the BCoV positive cattle

was from the Volta region (Fig. 2c).

Sequencing of the RdRp region

Sequencing was performed on the partial RNA-dependent RNA polymerase (RdRp) region of the four

bovine coronavirus isolates. Figure 3 shows a cladogram constructed for all four bovine coronaviruses

identified in goats and cattle. Sequences obtained from this study formed one large monophyletic

group with those from different species and countries. The goat sequences were similar to each other

and clustered within the same clade. Hence, no major variations were observed with our isolates and

those from elsewhere.

Discussion

This is the first report on molecular detection and phylogenetic analysis of BCoV infection in both

cattle and small ruminants in Ghana. The molecular prevalence of BCoV was 0.3%. Among the

positive cases, 0.2% were samples obtained from the Upper East region whereas 0.1% was from the

Volta region. Greater Accra and Northern regions did not record positive cases. Strikingly, upon

stratification by animal type, three out of the four BCoV positive cases were goats whereas one was

cattle. No case was recorded in sheep.

The prevalence of BCoV in this study is lower compared to previous studies. A study by Lojkić et al. in

Croatia (2015) found 82 of the 101 analyzed fecal and three nasal samples (81%) to be positive for

BCoV by RT-PCR [9]. Another study by Kumar et al. in India reported 9.38% (15/160) prevalence of

BCoV in cattle [23]. The higher prevalence in these studies could be due to the fact that they considered only cattle that were symptomatic, presenting with diarrhea. This potentiates the likelihood of obtaining samples that are positive for BCoV compared to large-scale randomized screening used in this study. Studies by Lathrop et al. [24] and Cho et al. [25], and Hasoksuz et al. [26] also reported a similarly high prevalence of BCoV in the USA. Other factors that could account for the lower prevalence in this study are the disparities in geographical location and timing of sampling relative to viral shedding. Of note, BCoV persists longer in lower temperatures and therefore is able to remain active in the environment all year round in temperate regions compared to a tropical region. Additionally, differences in animal management systems between developing countries such as Ghana and the developed countries could account for the differences in prevalence rates.

Smith et al. indicated that farm management systems have a significant influence on BCoV infection rate [27]. In Ghana, the extensive and semi-intensive systems are largely practiced in cattle, sheep, and goats rearing. On the other hand, the feedlot system of management is a commonly practiced system in most developed countries. This system is a variant of intensive farming practice where animals are confined throughout the year. Such relatively close confinement could enhance the transmissibility of BCoV infection within herds or flocks, thus increasing the detection rate of the virus. Furthermore, BCoV infection is generally self-limiting [28]. Viral shedding is transient and is known to last for only about 9 days. Given that sampling of this study was randomized, with almost all the animals included being asymptomatic, it is possible that sampling was done at a time when viral shedding had terminated.

In Ghana and many developing countries, different animals live in proximity to one another. There is thus the need to investigate cross-species infection because the close and sustained interaction between different animals poses an increased risk of spillover of communicable diseases between animals. However, globally, there are only a few studies evaluating the prevalence of BCoV in non-cattle livestock [14–18]. The presence of BCoV in three goats out of the four BCoV positive cases, thus, indicates a possible active infection and provides update information of spillover of BCoV from cattle to small ruminants in Ghana. It is instructive to state that in Ghana mixed farming (livestock) is

what is largely practiced by farmers and this predispose animals to cross-species infections.

Comparison of the nucleotide sequences obtained in this study with sequence data contained in the

National Center for Biotechnology Information (NCBI) GenBank revealed no major differences. Three

of the four bovine coronavirus isolated were found in goats and one from cattle. Sequences obtained

from the goats (Ghana Goat 1–3) were similar to each other and clustered within the same clade.

Likewise, the sequence obtained from cattle (Ghana cattle) was also similar to those from other

countries. Hence, no major variations were observed with our isolates and those from elsewhere.

However, this finding corroborates our previous deposition of the possible interspecies transmission of

BCoV from cattle and wildlife to small ruminants [19].

Conclusion

Given that Ghana predominantly practice the extensive and semi-intensive systems of animal rearing,

our study highlights the potential for spillover of BCoV to small ruminants in settings with mixed

husbandry and limited separation between species. Continuous surveillance of BCoV in the country is

thus critical to avert future surge and probable increase in host range.

List Of Abbreviations

BCoV: bovine coronavirus; RdRp: RNA-dependent RNA polymerase; RT-PCR: reverse transcriptase

polymerase chain reaction; NCBI: National Center for Biotechnology Information.

Declarations

Ethics approval and consent to participate

This study was approved by the Wildlife division of the Ghana Forestry Commission (Approval number:

AO4957). Written informed consent was obtained from all owners whose animals were used in the

study.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding

author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Authors' contributions

CD, YAS, AS and MO designed the study, supervised the research and laboratory analysis, drafted and revised the manuscript. VB was involved in the collection of data, laboratory analysis, drafting and revision of the manuscript. PED, RY, JL, YOF, OA, BE, WT, SO and CA were involved in the collection of data and laboratory analysis. EWO and PED was involved in the data analysis and interpretation, drafting and revision of the manuscript. RF was involved in the drafting and revision of the manuscript All authors read and approved the final manuscript.

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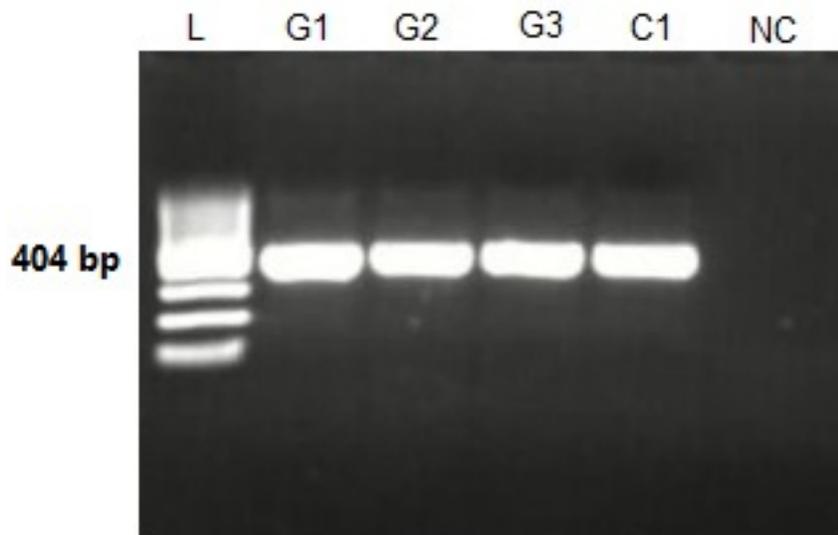
Figures

Figure 1

Gel image of the Hemi-nested PCR products used for sequencing. L: 1000 bp ladder; G1: goat 1; G2: goat 2; G3: goat 3; C1: cattle 1; NC: negative control
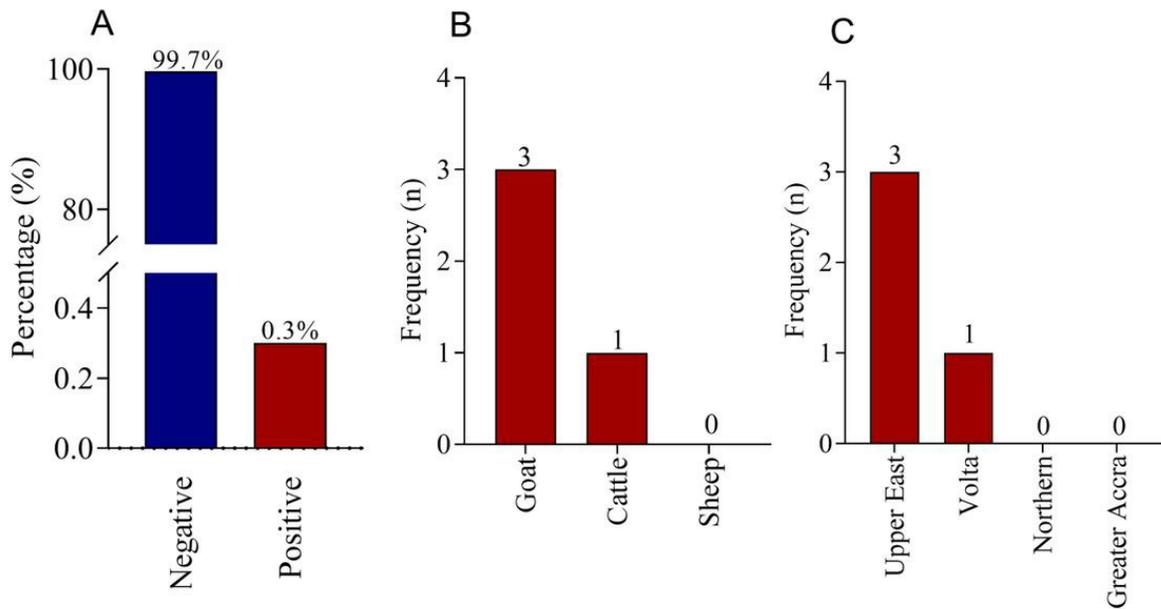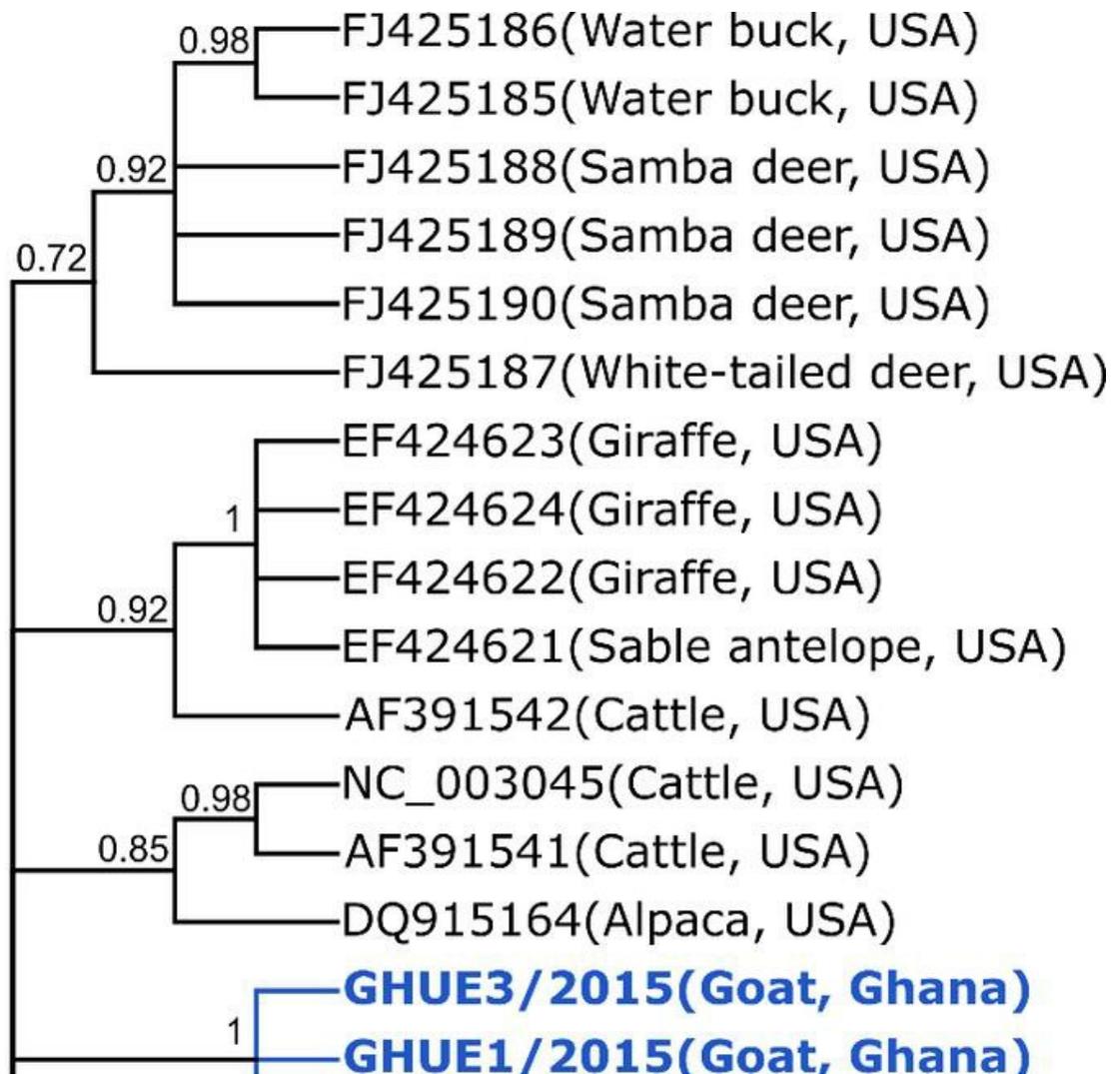
Figure 2
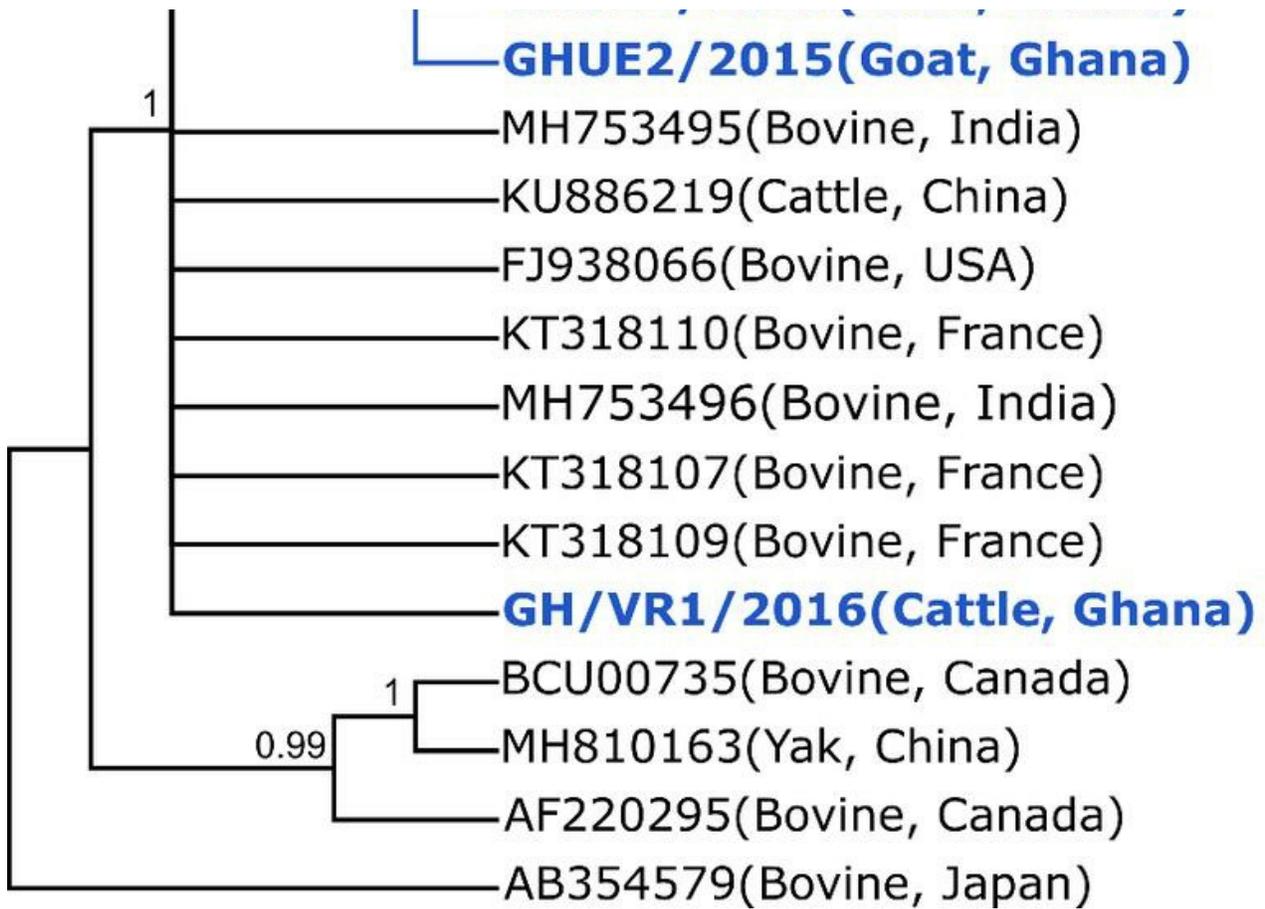
The prevalence of BCoV.

Figure 3

Bayesian majority-rule cladogram comparing partial and full length BCoV RdRp obtained in the study to those from different species and countries.