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Prevalence of rotavirus group A and associated genotypes detected in calves in Southern Highlands and Eastern Tanzania

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Rotavirus group A (RVA) causes neonatal calves diarrhoea, causing massive economic losses in many countries due to deaths, treatment costs, and stunted growth. However, little is known about Tanzanian cattle's RVA burden and genotypes. The prevalence and genetic diversity of rotaviruses circulating in cattle in Southern and Eastern Tanzania regions were investigated. A total of 272 faecal samples collected from calves (102 from Mbeya, 89 from Iringa, and 81 from Morogoro) were tested for RVA. 30 (11.03%) of the 272 samples tested positive for RVA, 7 (6.8%) of the 30 RVA positive samples were from Mbeya, 23 (28.39%) from Morogoro, and no positive samples were found in the Iringa region. Using RT-PCR, 20 samples were identified as G10 genotypes, while the remaining 10 samples were untypeable with the primers used, whereas all P genotypes were untypeable. To confirm the RT-PCR results, representative samples were chosen and sequenced in the ABI 3130XL using a Big-dye Terminator kit with Sanger dideoxy sequencing method. Phylogenetic analysis and nucleotide comparison revealed that G10 was closely related to and clustered with human G10 strains from neighboring Kenya, implying that circulating G10 strains may have the potential for zoonotic transmission. When circulating G10 amino acid alignment was compared to bovine vaccine strain B223, our G10 samples had various nucleotide substitutions at antigenic epitopes, which resulted in the acquisition of glycosylation sites suggesting that G10 has the potential to evade neutralization antibodies induced by a vaccine. Our study provided preliminary data on RVA in cattle in Tanzania, paving the way for further research into the rotavirus epidemiology and risk factors associated with RVA in cattle in Tanzania.

Key words: Cattle, rotavirus groups A, G10 genotype, Tanzania.

INTRODUCTION

Rotavirus group A (RVA) is the causative agent of diarrhea-related cases and mortality in children under the age of five and young animals worldwide (Liu et al., 2012; Komoto et al., 2016; Troeger et al., 2018). The RVA has been found in a variety of animal species, including cattle, pigs, dogs, horses and other species (Martella et al., 2010: Li et al., 2016; Papp et al., 2013). In cattle, responsible for neonatal calves' diarrhea, causing massive economic loss in many countries due to deaths, treatment costs, and stunted growth (Bellinzoni et al., 1989; Garaicoechea et al., 2006; Bassera et al., 2010). Some countries have implemented vaccine programs to combat rotavirus infection in cattle due to its significant economic impact (Rodrguez-Limas et al., 2009; Swiatek et al., 2010). However, there is no RVA vaccination program in place in Tanzanian cattle, despite the fact that it has been effectively implemented in children under five since 2013 (Moyo et al., 2011). A few studies have examined the prevalence of RVA in African nations. These studies found that the RVA prevalence was 13.1% in the Ivory Coast (Yahaya et al., 2018), 3.64% in Ethiopia (Debelo et al., 2021), 1.8% in Nigeria (Garba et al., 2020) and elsewhere, 8.8% in Korea (Chae et al., 2021), as well as 5.11% in Bangladesh (Barua et al., 2019). In Tanzania, a cattle is one of the major source of protein and a significant source of income. The cattle population is rapidly growing; it was estimated to be 19 million cattle in 2010 and has recently increased to 28 million cattle by 2020 (Statista, 2022). Despite the significant contribution of cattle to the national economy, little is known about the burden, prevalence and genotypes of RVA in cattle in Tanzania. Because of the potential impact of rotavirus in cattle, understanding the burden and genotypes circulating in cattle is critical in protecting cattle health and thus improving human livelihood.

Several RVA strains are zoonotic and have been linked to a number of diarrhea cases and deaths in children under the age of five (Pecenka et al., 2017; Troeger et al., 2018). In Bangladesh, a bovine-like RVA was discovered in children suffering from diarrhea (Afrad et al., 2013). Because humans and animals coexist in Tanzania, there is a risk of zoonotic transmission to humans. Zoonotic transmission is known to drive rotavirus diversity, which may have implications for vaccine performance (Doro et al., 2015; Vlasova et al., 2017). As a result, highlighting bovine strain profiles and investigating their zoonotic potential is critical in examining the design of strategic controls for RVA intervention in both humans and animals.

The RVA virus is a double-stranded RNA virus in the Reoviridae family. Its genome is made up of 11 segments, six of which code for structural proteins (VP1-VP4, VP6 and VP7) and five of which code for non-structural proteins (NSP1-NSP5/6) (Estes and Kapikian, 2007). The VP4 and VP7 proteins are the most important because they form a dual classification system (VP7 forms G1.....Gnth genotypes and VP4 forms P[1],P[2].....P[nth] genotypes) and are used for eliciting independent immune responses, making them crucial in vaccine development (Matthijnssens et al., 2008).

In humans and animals, 41 G genotypes and 57 P genotypes have been identified so far (RCWG, 2022). The most common G genotypes found in cattle are G6, G10, and G8, while the most common P genotypes are P [5], P[11], and P[1] worldwide. Other genotypes detected sporadically include G types G1-G3, G5, G11, G15, G17, G21, and G24) and 11 P types P[3], P[6], P[7], P[14], P[17], P[21], P[29], and P[33], with G6P[5], G6P[11], and G10P[11] being the most common (Papp et al., 2013).

Because of RVA infection in cattle reported to have economic drawbacks and limited studies on the RVA circulating in cattle in Tanzania, this study was carried out to better understand the RVA burden and genetic relationship of rotavirus genotypes circulating in cattle in Tanzania. The study was conducted in a selected region with a large cattle population and animals-human interaction found in the Southern highland (Mbeya and Iringa) and Eastern (Morogoro) Tanzania.

MATERIALS AND METHODS

Description of the study area and design

This cross-sectional study was carried out in three Tanzanian districts, namely Mbarali, Kilolo, and Mvomero, which were chosen for their large cattle populations and animal-human interaction from three regions, namely Mbeya, Iringa (Southern highlands), and Morogoro (eastern Tanzania). Between April 2019 and May 2020, samples were collected. Mbarali is located between latitudes S07" 41' and S09" 25' and longitudes E33" 40' and E35" 40' above sea level, at an elevation of 1000 to 1800 meters (masl). The cattle population was estimated to be 198,316 (HWMB, 2020). In the Iringa region, the Kilolo district is located between latitudes 35" 54' E and 35" 57' E and longitudes 8" 01' S and 8" 06' S. there are 79500 cattle (HWKI, 2020). Mvomero is situated between 6'07' and 7'05' South and 37'17' and 37'65' east. The district altitude ranges from 300 to 400 masl in Turiani, Mvomero, and Mzumbe divisions to 1000 to 1700 masl in Mgeta division. The cattle population was estimated to be 125988 (HWMV, 2020).

Ethical considerations

The permit on the subject of animals was obtained from Sokoine University of Agriculture via the CVMBS College Research

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Innovations and Publication Committee (SUA/CVMBS/R.1/2019/1). Permission was also granted by the Regional Administrative Secretary (RAS), District Administrative Secretary (DAS).

Sample collection

The aseptic collection of feces was done on calves younger than six months. Directly taken sample from the rectum of chosen calves, either with or without diarrhea. Approximately a pea-size feacal sample was picked from the animal and placed in duplicate into sterile cryogenic vials (Corning, Lowell, USA) and stored in liquid nitrogen. The vials contained 1 ml Trizol reagent (Life Technologies Corporation, Carlsbad, USA) and 1 ml of viral transporting media (Remel Micro test M6, Lenexa, USA). Samples transported in liquid nitrogen to Sokoine University of Agriculture. On arrival, samples were stored in a -80 ^{0} C freezer (NUAIRE, Plymouth, USA).

Sample preparation and RNA extraction

Samples were removed from the ultralow temperature freezer and placed in a class II biological safety cabinet (SterilGard, Sanford, USA) allowing them to thaw. Afterward, samples were centrifuged at 5000 g for 8 min using Centrifuge 5717R (Eppendorf, Hamburg, Germany). Then 250 µL of supernatant was harvested and used for RNA ex-traction using direct zol-RNA MiniPrep Kit (Direct zol RNA MiniPrep, Tustin, United States of America) following the manufacturer's instructions. The final volume of dsRNA after extraction was 50 µL, 25 µL of the total volume was aliquoted into another PCR tubes and stored in -80^{0} C ultra-freezers and one was used for cDNA synthesis.

cDNA synthesis

The dsRNA was first denatured at 97^{0} C for 5 min and chilled on ice for 2 min, 8 µl of the denatured RNA was used for cDNA synthesis using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, USA) manufacturer's instructions. The final volume of the cDNA was 20 µl which was divided into two aliquots of 10 µl and stored at -80 ^{0} C.

Detection of Rotavirus group A

Rotavirus group A (RVA) was detected using primers (Macrogen, Seoul, Republic of South Korea) NSP3-F and NSP3-R yielding 87 bp on gel electrophoresis as described previous (Amimo et al., 2015). The cDNA obtained was used to detect RVA using the Invitrogen Platinum Taq kit (Invitrogen, Carlsbad, USA) following the manufacturer's instructions.

Briefly, the PCR master mix had 2.5 µL 10X PCR Buffer, 0.75 µL 50 mM MgCl2, 0.5 µL 10 mM dNTP, 0.1 µL Platinum Taq DNA polymerase, 1 µL 10 µm NSP3 forward primer, 1 µL 10 µm NSP3 reverse primer and 18.15 µL molecular grade water. Then 1µL of DNA template, RNA extraction control, positive control (Rotarix Vaccine and Bovine rota-virus Indiana strain) and PCR control (molecular grade water) as negative control were added to the respective PCR master mix. The Gel electrophoresis was performed on the PCR product using 3% agarose gel (AMRESCO, Solon, USA) stained with gel red nucleic acid stain (Biotium, Fremont, USA) at 110 voltage for 1.30 hr. The DNA marker (Bionexus, Oakland, USA) of 15 µL was loaded along with the PCR product. The gel visualization of amplicon size was performed using Dual UV Trans-illuminator (Analytic Jena US, Upland, USA).

Genotyping of Rotavirus group A

The G and P genotyping was performed by multiplex reverse Transcriptase nested PCR using primer sets and amplification conditions as previously described (Ansari et al., 2013). The Invitrogen Platinum Taq kits were used for PCR amplification following manufacture instructions. The primer set (VP7-F and VP7-R) for round 1 PCR target the VP7 gene for all RVA resulting in a band size of 881 bp on gel electrophoresis. The primer sets for round 2, multiplex PCR for G typing targeted G1, G2, G3, G4, G8, G9, G10 and G12 Whereas for the P typing, the primer set (Con3 and Con2) (Ansari et al., 2013) for round 1 targeted the VP4 gene for RVA which produces a band size of 876 bp on gel electrophoresis. The round 2 targeted P[4], P[6], P[8], P[9], P[10] and P[11] (Ansari et al., 2013). Gel electrophoresis was performed using a 2% agarose gel stained with gel red at 110 voltages for 1.30 hr. The gel visualization of amplicon size was done using a gel documentation system.

Sequencing of VP7 gene

To confirm RT-PCR results and for determination of genetic and antigenic relationship of the RVA genotypes, the PCR product for round one RT-PCR for VP7 were sent for sequencing at the Macrogen in Netherland. The PCR product were sequenced using Sanger dideoxy sequencing method on ABI 3130XL machine (Applied Biosystems, Carlsbad, USA) using a BigDye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, USA). The same primer used in round 1 was used for sequencing.

Sequence processing

The ABI sequence files of the forward and the reverse sequences were edited aligned and consensus sequences generated using the BioEdit sequence alignment Editor 7.2.5. The rotavirus genotypes were assigned based on the nucleotide percentage identities using the ViPR (ViPR, 2021) based on the RotaC algorithm developed by Maes et al. (2009) and BLAST. The cutoff value for genotypes assignment was 82% nucleotides identities for both VP7 and VP4 typing (Matthijnssens et al., 2008).

Phylogenetic analysis and amino acid alignments

Using MEGA X, the best fit substitution model was identified based on the lowest BIC scores (Bayesi-an information Criterion). Tamura 3-parameters T92 (BIC 2695) models were found to best fit the data (Nei and Kumar, 2000; Kumar et al., 2018). Therefore, the phylogenic tree was constructed using the Maximum likelihood method and the Tamura 3-parameters with 1000 bootstrap replicates (Kumar et al., 2018; Tamura, 1992). The reference sequences for the analysis were found by blasting our study sequence nucleotides Blast and the translation done by blastx on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The accession numbers for strains representing lineages were obtained from previous publications and nucleotide retrieval was performed using nucleotide blast. The alignment of deduced amino acid was performed using BioEdit sequence alignment Editor 7.2.5.

RESULTS AND DISCUSSION

Prevalence and genotypes distribution of RVA in calves

A total of 272 fecal samples (102 from Mbeya, 89 from

Table 1. Site infection rate of RVA in calves.

Region	District	Ward	Village	N=272	Rotavirus positivity rate (%)
Mbeya	Mbarali	Mawindi	Itipingi	9	4 (44.44)
			Kangaga	9	1 (11.11)
			Manienga	8	0
		Chimala	Igumbilo	5	0
			Mwale	9	0
		Ihai	Ihai	14	1 (7.14)
			Kibaoni	2	0
		Luhanga	Luhanga	46	1 (2.17)
Morogoro	Mvomero	Mhondo	Kichangani	6	5 (83.33)
		Manyinga	Diongoya	8	3 (37.5)
		Mtibwa	Lukenge	20	8 (40)
		Melela	Kibaoni	26	4 (15.38)
		Mangai	Malandizi	21	3 (14.25)
Iringa	Kilolo	Ihimbo	Utengule	9	0
			Itarula	11	0
			Ihimbo	13	0
		Ng'uruwe	Lukani	12	0
			Ng'uruwe	11	0
		Image	Image 8	11	0
			Uhominyi	6	0
			Ilawa	7	0

Source: Authors

Iringa, and 81 from Morogoro) collected from calves were tested for RVA, with 30 (11.03%) samples showing a band size of 87 bp on gel electrophoresis. The 7 (6.8%) of the 30 RVA positive samples were from Mbeya, 23 (28.39%) from Morogoro, and no positive samples were found in the Iringa region. The RVA positivity rate for the sites was shown in Table 1. The results show that the infection rate varies by region, with lower prevalence in Mbeya and Iringa than in Morogoro possibly due to sample collection criteria of testing symptomatic and asymptomatic calves. The results were comparable to the prevalence of RVA reported in other African countries such as Ivory Coast (Yahaya et al., 2018), Tunisia (Fodha et al., 2005), Nigeria (Babalola et al., 20200. But also lower prevalence compared to this study have been reported in Nigeria (Garba et al., 2020), Ethiopia (Debelo et al., 2021). Also the results were comparable to other countries outside Africa such as Australia (Swiatek et al., 2010), India and Mexico (Rodrguez-Limasa et al., 2009; Basera et al., 2010). However, six European countries have reported a higher prevalence of RVA in cattle (Midgley et al., 2012). The prevalence could not be compared to other neighboring countries due to the lack of information on RVA infection in cattle from other East African countries. Infection with rotavirus in cattle was first documented in this study. Bovine rotavirus diarrhea is a high morbidity condition that costs cattle herders significantly in terms of treatment expenses and decreased weight gain in infected animals (Bartels et al., 2010). This study offered crucial details about the prevalence of rotavirus infection in cattle in Tanzania. Unwrap for additional research on the prevalence of RVA in cattle and the risk factors that are linked to it. The study did have some limitations, though; it was unable to identify any additional pathogens that might be responsible for diarrhea. This may aid in locating the pathogens responsible for the diarrhoea in Tanzanian cattle. Additionally, only a small number of positive samples were found, making it impossible to link the presence or absence of diarrhea in calves with RVA in those animals.

Due to the failure of other samples to amplify in rounds one and two of G and P typing RT-PCR, only two samples were able to be sequenced for VP7 and none for VP4. These untypeable G and P may not have amplified in round one of P typing RT-PCR and some G typing RT-PCR due to primer failure caused by mutation or insufficient nucleic acid materials (Gómara et al., 2001; 2004). Only the G10 genotype was found in cattle circulating in Mbarali and Mvomero, along with untypeable

P genotypes. The G10 genotype has been identified as one of the most common genotypes circulating in cattle in several African countries, including Ivory Coast, Morocco, and Nigeria (Monney et al., 2019; Babalola et al., 2020; Amine et al., 2020). The G10 reported has been linked to numerous cases of diarrhea in calves (Badaracco et al., 2013; Monney et al., 2019). The G10 is the second-most common genotype worldwide, accounting for 20% of the circulating genotypes in calves (Papp et al., 2013). However, they also make up 20% of the circulating genotypes in many different countries (Castells et al., 2020). It has frequently been reported in combination with P [11] (Medeiros et al., 2019; Hossain et al., 2020; Castells et al., 2020), however, P[11] was not identified in this study. Since G10 genotype is common and has a significant impact on health in many nations, some vaccines to treat calf diarrhea now contain G10 in their formulation. For instance, the BOVILIS® GUARDIAN® (GUARDIAN®, Merck Animal Health, USA) vaccine's cocktail includes the rotavirus strains G10 and G6 as well as types 1 and 3 of coronavirus, types C and D of Clostridium perfringens, and type K99 of Escherichia coli (Papp et al., 2013; Karayel et al., 2017). Primers were used in this study to genotype P [4], P[6, P[8, P[9], P[10], and P[11] P genotypes. Other P genotypes, such as [P6], P [14], and P [15], have been reported in conjunction with G10 (Hossain et al., 2020; Babalola et al., 2020). The P genotypes were all untypeable, which could be due to primer failure due to mutation that why could not amply in round one of P genotyping RT-PCR and only 2 samples yield a PCR product for round one and two of G genotyping RT-PCR or different strain not recognized by used primer (Gómara et al., 2001; 2004). The study was unable to obtain sequence data for the corresponding P genotype, and as a result, it was unable to fully document the genetic diversity of G10.

The findings provide preliminary evidence of G10 genotype for the first time in Tanzania; therefore, additional research is required to identify other rotavirus genotypes in cattle in Tanzania for better development of control programs including vaccination. The sequences of the G10 genotypes have been deposited in the gene bank and assigned the accession numbers MW718929 and MW718930.

Phylogenetic analysis VP7 gene

The bovine strains were phylogenetically related (97%) and belonged to lineage II. The authors Bovine G10 clustered with G10 strains isolated from humans in neighboring Kenya (Figure 1), with nucleotide identities ranging from 97.13% to 97.89% shared suggesting their potential of G10 genotypes for zoonotic transmission. In symptomatic African children and elsewhere, a number of bovine-human G10 and human-bovine-like reassortant strains have been identified (Esona et al., 2011). However, more research into the genomic properties of these genotypes is required to understand the possibility of reassortment events and their origin. Interspecies and reassortment events are known to contribute to the variety of rotavirus strains that affect humans (Nakagomi and Nakagomi, 2002; Gomara et al., 2004; Martella et al., 2006; Rajendran et al., 2014) in which higher strain diversity may have an impact on vaccine effectiveness. Understanding these events is therefore critical in designing effective strategic control programs. Other African countries that have reported G10 genotypes in cattle include South Africa, Mozambique, and Ivory Coast (Monney et al., 2019; Strydom et al., 2021), but the majority of their G10 strains are from lineage V (Strydom et al., 2021). Our strain clustered distantly from vaccine strain B223 in lineage IV, implying that the circulating strains G10 is may be antigenically distinct.

Amino acid analysis

Amino acid substitution was discovered at the antigenic region of the circulating G10 strain compared to the bovine vaccine strain B223 on amino acid alignment (Table 2). The antigenic region variation has been linked to the ability of the wild type strain to evade neutralization antibody induced by the vaccine strain (Hoshino et al., 2004; Aoki et al., 2009; Trinh et al., 2007; Banyai et al., 2009; Harastani et al., 2020). However, more research on analysis is required to include a large number of G10 strains from across the country in order to make a conclusive observation. In our strain RVA/Cow-wt/TZA/Morogoro-RC017/2019/G10P[x], the amino acid asparagine (N) at position 96 was replaced by serine (S), resulting in the acquisition of glycosylation site at 94 (NDS). Other glycosylation sites at 69 (NAT) were found to be conserved in bovine vaccine strain B223. Similar findings were reported in Ghanian G10 strains (Badaracco et al., 2013). The glycosylation of amino acid residues has been reported to increase virus pathogenicity and resistance to monoclonal antibody by covering the virus with polysaccharide thus hiding the protein part of the virus (Caust et al., 1987; Ciarlet et al., 1994; 1997).

Conclusion

For the first time, rotavirus infection in Tanzanian cattle was reported in the study; therefore, there is a need for ongoing monitoring of rotavirus infection in cattle, as well as for awareness raising and an evaluation of the financial costs and potential effects on public health. Our bovine genotypes clustered with a human strain from a neighboring country, indicating the possibility of zoonotic transmission to human; however, further research into their genome properties is required to understand

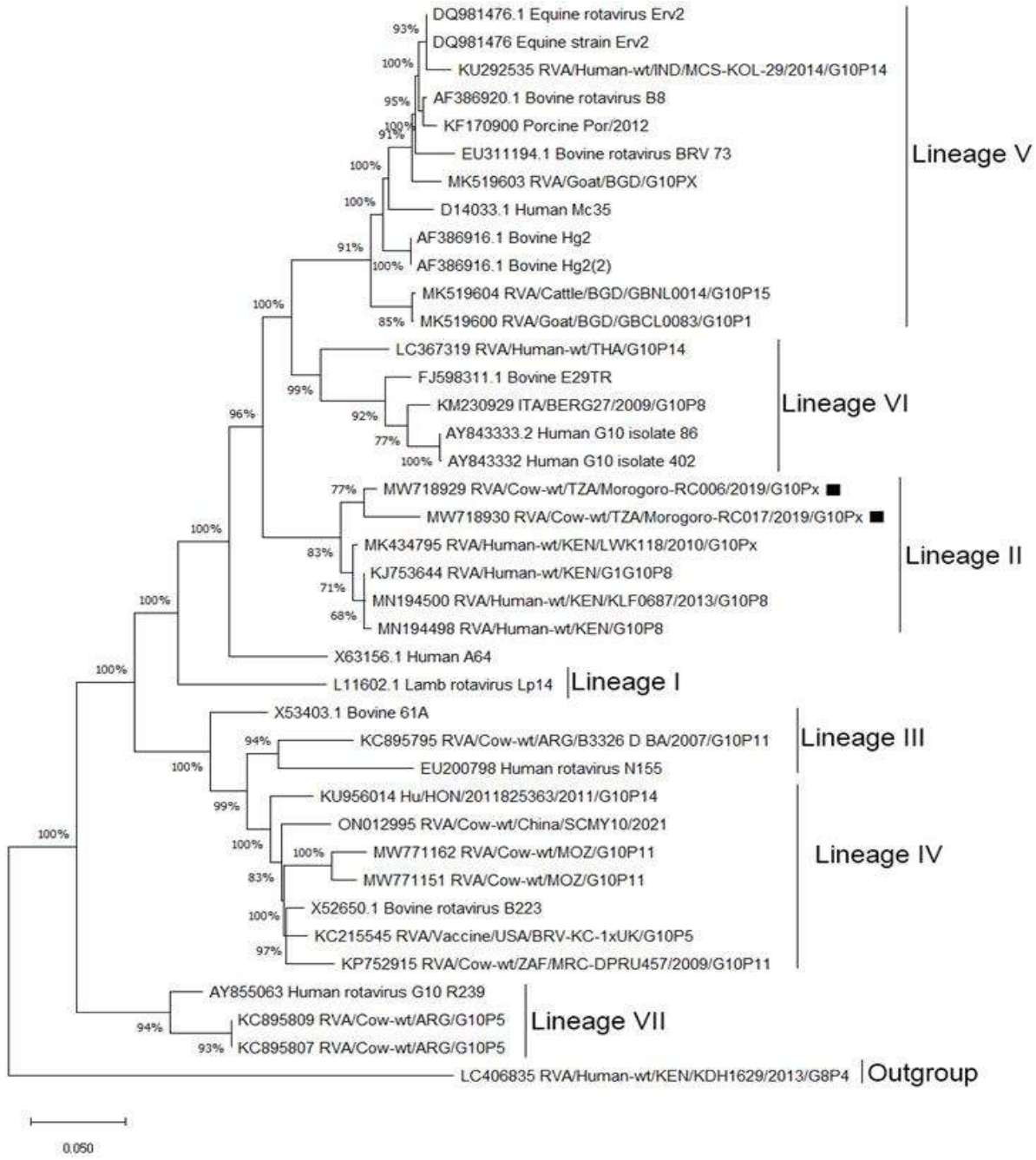

Figure 1. The nucleotide sequence phylogeny tree depicting the genetic relationship of VP7 gene of G10 genotypes, this study isolates are labelled with a black filled square.
Source: Authors

reassortment events and interspecies transmission. The occurrences of amino acid variation at the antigenic epitopes and N-linked glycosylation sites in comparison to the reference strain suggest potential of G10 genotype for escape neutralization antibodies. Because only a few genotypes were studied, and further research was recommended to gain a better understanding of rotavirus epidemiology, ecology, and evolution.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Table 2. The antigenic residues of VP7 (7-1a, 7-1b, and 7-2) present in genotype G10 strains of reference strain B223 and circulating strains were compared. Green highlights the amino acid residues in the circulating strains that differed from those in the reference strain.

	LN	7-1a																														
		87	91	94	96	97	98	99	100	104	123	125	129	130	291	201	211	212	213	238	242	143	145	146	147	148	190	217	221	264
Bovine vaccine strain B223	2	T	T	N	N	E	W	T	S	Q	D	A	V	D	K	Q	N	T	R	D	A	R	N	S	S	L	S	E	A	G
RVA/Cow-wt/TZA/Morogoro-RC006/2019/G10P[x]	9	T	T	N	N	E	W	T	S	Q	D	T*	V	N*	I*	Q	N	T	G*	D	A	R	N	S	S	L	S	E	A	G
RVA/Cow-wt/TZA/Morogoro-RC017/2019/G10P[x]	9	T	T	N	S*	E	W	T	S	L*	D	T*	V	D	I*	H*	N	T	G*	D	A	R	N	S	S	L	S	E	A	G

Source: Authors

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