Group A Rotavirus Genotypes among Calf Herds in Southwest Nigeria and Implications for Human Rotavirus Vaccines' Efficiency

Michael Oluyemi Babalola

Epidemiology, Molecular Virology, and Special Pathogens Research, Department of Microbiology, Adekunle Ajasin University, Akungba Akoko, Ondo state, Nigeria
Email: michael.babalola@aaua.edu.ng

Abstract

Rotaviruses have been widely reported to be associated with diarrhea in humans but fewer studies abound on other mammalian species. This prospective study was conducted to detect and characterize Rotaviruses from freely ranged migratory herds of cattle in Ekiti and Ondo states, Nigeria with a view to further expanding knowledge on rotaviruses, possible animal-human interspecies transmission and impacts on vaccine efficiency. By convenience sampling, between September 2014 and February 2015, stool samples from 120 calves, comprising settled and migratory herds of cattle were obtained and examined for group A rotaviruses using Certest® Quadruple Enzyme Immuno Assay. Rotavirus genomes were isolated by extraction from the positive samples, reverse transcribed and amplified by One-Step reverse transcription polymerase chain reaction (RT-PCR), and genotyped by semi-nested multiplex PCR. Representative PCR products of the genotyped samples were purified and sequenced using Sanger method. The generated query sequences were queried to the GenBank to retrieve similar sequences for pairwise alignment by ClustalW. Phylogenetic analyses by Neighbour-joining method were conducted at 1000 bootstrap replicates to obtain phylograms using MEGA 6 software. Fifteen samples (15/120: 12.5%) were positive for rotavirus. No statistically significant association existed between rotavirus infection and gender of the calves ($\chi^2 = 0$, df = 1, $p = 1$). Eight samples (8/15: 53.3%) were successfully genotyped where the G1, G5, G9, G10, G12, and P[6] were detected. Nucleotide Sequencing of the VP7 and VP4 genes of the genotyped samples confirmed strains G1P[6], G10P[6], and G12P[6] with 58% - 100% nucleotide identity within these viruses. Phylogenetic analysis revealed 2 possible transmissions from India and Honduras. Bovine rotaviruses were detected in freely ranged and settled calf herds in southwestern Nigeria at a rate of 12.5%. The strains

identified were analysed to be group A rotavirus strains with potential interspecies transmission from human to calves and from bovine to humans. The detected mixed strains could eventually impact negatively on the effectiveness of available rotavirus vaccines over the prevailing serotypes in human infections.

Keywords

Group A Rotavirus, Diarrhea, Calves, Genotypes, Vaccines

1. Introduction

Rotaviruses are the most important infective etiology of enteric disease manifesting in diarrhea, severe dehydration and eventual death of a wide range of juveniles of humans, domestic and wild animals in the developing countries, while it is a significant cause of morbidity in the industrialized countries [1]. Rotavirus groups A, B and C are responsible for infections in mammalian species of which the Group A rotaviruses (RVAs) are the most important group associated with infection in a wide range of animals including humans [2]. The genus Rotavirus of the family Reoviridae is presently classified according to serological and genetic features into ten approved groups A to J of which group A Rotavirus (ICTVdb No. 00.060.0.03.001) is recognized as the most common etiologic agent of severe dehydrating diarrhea in infants and young children in both developed and developing countries [3] [4] [5] [6].

Group A rotaviruses were reported to be the major causes of severe diarrhea in young children and animals around the world, affecting nearly all animals from whales and snakes to cows and pigs, resulting in dehydration and death. Bovine rotavirus diarrhea is a high morbidity condition causing important economic losses for cattle herders in terms of treatment cost and reduction of weight gain in infected animals [7]. Previous studies have shown the evidence of human rotaviruses with genomic segments of bovine rotaviruses as a result of reassortment or direct transmission to human [8]. Rotavirus diarrhea constitutes drawbacks in livestock farming, particularly in bovine calves aged below 1 year and contributes significant epidemiological problem in human-animal disease transmission, prevalence, monitoring and control.

In Nigeria, significant studies have been carried out on Rotavirus infection of humans, while very few studies have been carried out on Rotaviruses in animals. Among these, [9] reported a prevalence of 3.2% among calves below 56 days old in North-Eastern Nigeria, while a prevalence of 23% was reported in cattle herds of Zaria, Nigeria [10]. However, bovine Rotavirus studies in southern Nigeria remained unexplored. This study was therefore designed to detect bovine group A rotaviruses from bovine calves in selected areas of Ondo and Ekiti states, to further define the epidemiology and molecular characteristics of rotaviruses from animal sources in Nigeria.

2. Methodology

2.1. Collection of Fecal Samples

By convenience sampling, one hundred and twenty (120) fecal samples were aseptically collected from diarrheic calves aged less than 1 year, by herd to herd visits of settled and migratory cattle herds in Ondo and Ekiti states, Nigeria between September 2014 and February 2015. Eighty samples (80) were from males while 40 were from females.

2.2. Detection of Rotaviruses Using Enzyme Immuno-Chromatographic Assay

The fecal samples were immediately screened for the presence of group A rotavirus or transported to the laboratory on ice and stored at −20˚C until tested. The fecal samples were tested for Rotaviruses using the Certest EIA kit according to the manufacturer's instructions and as previously reported [11].

2.3. Preparation of Stool Suspension from Rotavirus Positive Samples

For each of the 15 samples that were positive for rotaviruses, 10% (v/v) fecal suspensions were prepared in sterile Phosphate Buffered Saline, PBS (pH 7.2) after vigorous mixing and clarified by centrifugation at 3000 g for 20 min at 4˚C [12]. The resultant supernatants were harvested, divided into aliquots and stored at −80˚C until use for the downstream molecular assays for group A rotaviruses at the Department of Virology, University of Ibadan, Nigeria.

2.4. Extraction of Rotavirus Double Stranded RNA (dsRNA) Genome

For each aliquot of the 15 rotavirus positive samples, rotavirus dsRNA genome was extracted from the 10% stool suspension using the Total RNA Purification kit (Jena Bioscience GmbH, Jena Germany) according to the manufacturer' instructions.

2.5. Reverse Transcription and Amplification of the VP7 Gene Segment of Rotavirus dsRNA Genome by One-Step Reverse Transcription Polymerase Chain Reaction (One-Step RT-PCR)

First round One-Step Reverse Transcription and Amplification of the dsRNA genome targeting the full length (1062 bp) VP7 gene segment 9 encoding the VP7 Glycoprotein was performed using sBeg9 and RVG9 primers (Eurofins, Germany) with the invitrogen One-Step RT-PCR reagent kit (Invitrogen Life Technologies, Carlsbad USA) following the manufacturer's instructions and a previous method with minor modifications at the cycling conditions in Table 1 [13]. Synthesized dsDNA amplicons of the target sequence were then utilized as template in the second round semi-nested PCR for genotyping the detected rotaviruses.

2.6. VP7 Genotype Characterization (G-Typing) of Bovine RVA by Semi-Nested Multiplex PCR

The VP7 G-Typing assays were conducted on each of the synthesized first round amplicons of the detected rotaviruses using a consensus anchor primer RVG9 along with type specific cocktail of forward primers (Eurofins, Germany) for G1 (aBT1), G2 (aCT2), G3 (aET3), G4 (aDT4), G5 (aFT5), G6 (aDT6), G8 (aAT8), G9 (aFT9), G10 (mG10), G11 (aBT11), G12 (G12) genotypes as previously described [13] [14] [15]. Semi-nested PCR reaction (Table 2) was conducted using the 5X Red Load Taq Master High Yield PCR Mix (master mix of thermostable DNA polymerase, dATP, dCTP, dGTP, dTTP, KCl, MgCl$_2$, red dye, gel loading buffer and stabilizers) (Jena Bioscience GmbH, Jena Germany) following the cycling conditions in Table 1.

2.7. Reverse Transcription and Amplification of the VP4 Gene Segment of Rotavirus dsRNA Genome by One-Step Reverse Transcription Polymerase Chain Reaction (One-Step RT-PCR)

First round One-Step Reverse Transcription and Amplification of the dsRNA genome targeting the VP4 gene segment (876 bp) was performed using Con3 and Con2 primers with the invitrogen One-Step RT-PCR reagent kit (Invitrogen Life Technologies, Carlsbad, USA) following the manufacturer's instructions and a previous method with minor modifications, at the cycling conditions in Table 1 [16]. Synthesized dsDNA amplicons of the target sequence were then utilized as template in the second round semi-nested PCR for genotyping the detected rotaviruses.

2.8. VP4 Genotype Characterization (P-Typing) of Bovine RVA by Semi-Nested Multiplex PCR

The VP4 P-Typing assays were conducted on each of the synthesized first round amplicons of the detected rotaviruses using a consensus anchor primer Con3 along with type specific cocktail of primers (Eurofins, Germany) as previously described [15] [17]. Assays were performed for the P-genotype of each of the isolated first round amplicons, using a cocktail of the primers set (specific for genotypes P[4], P[6], P[8], P[9], P[10], P[11] and P[14]). Semi-nested PCR was conducted using the 5X Red Load Taq Master High Yield PCR Mix (master mix of thermostable DNA polymerase, dATP, dCTP, dGTP, dTTP, KCl, MgCl$_2$, red dye, gel loading buffer and stabilizers) (Jena Bioscience GmbH, Jena Germany) following the cycling conditions in Table 1.

2.9. Gel Electrophoresis and Documentation of the Multiplex PCR Amplicons for Determination of the Rotavirus VP7 and VP4 Genotypes

The second round multiplex PCR products were resolved by gel electrophoresis at 120 V for 60 mins in 1.5% Agarose gels containing 5 μl of 0.5 μg Ethidium bromide/ml along with a 100 bp DNA Molecular weight Standard (Jena Bioscience GmbH, Jena Germany) using freshly prepared 1X Tris Boric Ethylene Diamine

Table 1. Cycling Profile for first round One-Step RT-PCR Amplification of Rotavirus VP7 and VP4 Genes, and second round semi-nested Multiplex PCR for VP7 G-typing and VP4 P-typing in this study.

Parameter	Profile for first round One-Step RT-PCR for VP7 and VP4 target genes			Profile for second round semi-nested multiplex PCR for VP7 G-typing			Profile for second round semi-nested multiplex PCR for VP4 P-typing		
	Temp. (°C)	Time (min)	Cycle	Temp. (°C)	Time (min)	Cycle	Temp. (°C)	Time (min)	Cycle
RT	50	15'	01	NA	NA	NA	NA	NA	NA
PCR Activation	95	02'	01	94	02'	01	94	02'	01
Denaturation	94	0.30"	40	94	01'	40	94	01'	40
Annealing	45	0.45"	40	45	0.45"	40	45	01'	40
Extension	72	01'	40	72	01'	40	72	01'	40
Final Extension	72	07'	01	72	07'	01	72	07'	01
Hold	04	∞	-	04	∞	-	04	∞	-

Table 2. Reagent Mix for Bovine Group A Rotavirus VP7 G-typing by semi-nested multiplex PCR.

Reagent/concentration	Volume (µL) for 1 sample	
	Reaction X	Reaction Y
5X Red Load Taq Master High Yield PCR Mix	5 µL	5 µL
Forward primers:	0.5 µL of 20 pmol aBT1	0.5 µL of 20 pmol aFT5
	0.5 µL of 20 pmol aCT2	0.5 µL of 20 pmol aDT6
	0.5 µL of 20 pmol aET3	0.5 µL of 20 pmol mG10
	0.5 µL of 20 pmol aDT4	0.5 µL of 20 pmol aBT11
	0.5 µL of 20 pmol aAT8	0.5 µL of 20 pmol G12
	0.5 µL of 20 pmol aFT9	
20 pmol RVG9 Reverse Primer	0.5 µL	0.5 µL
PCR water	15.5 µL	16 µL
Total Aliquot of Mastermix	24 µL	24 µL
Template first round amplicon	1 µL	1 µL
Total reaction volume	**25 µL**	**25µL**

Tetra Acetic Acid (TBE) for agarose preparation and running buffer, in Owl® Gel Tank powered by a BIORAD® PowerPac. The resultant bands were visualized under ultraviolet light with the BIORAD® GelDoc system and documented. The specific G and P-genotypes were determined by the electrophoretic mobility bands and sizes (bp) of the amplicons in comparison to the molecular weight DNA Ladder [15].

2.10. Nucleotide Sequencing and Phylogenetic Analysis of the Detected Genotypes

Representative amplicons of the identified genotypes comprising the G1, G3, G9, G10, G12, and VP4 P[6] strains were randomly selected for nucleotide sequencing and investigation of their evolutionary relationships to those of other strains circulating in different regions of the world. The full length VP7 genes (1062 bp) and the partial VP4 genes (876 bp) of strains that could not be typed by multiplex semi-nested PCR were also selected for nucleotide sequencing. The amplicons were shipped to Macrogen Inc, Seoul, South Korea, for purification and sequencing in the forward direction with the respective specific genotyping primers stated above. The generated query sequences were extracted using the fasta format and edited using MEGA 6 software. Each query sequence was queried to the Gene-Bank Database by BLAST to retrieve reference sequences of the rotavirus genotype and aligned using the CLUSTALW program in MEGA 6 software. Pairwise distances of the sequences were estimated. The confidence values of the internal nodes in the phylogenetic trees were calculated by performing 1000 bootstrap replicates of the sequence alignment datasets, and the phylograms were constructed using the neighbor-joining method with MEGA version 6 [18].

3. Results

3.1. Prevalence of Rotavirus and Gender of the Bovine Calves

Fifteen fecal samples tested positive for rotavirus antigens from the 120 samples that were screened. This represents a total prevalence of 12.5%. Representative results of viral assay of the fecal samples are presented in Figure 1(a) and Figure 1(b). Appearance of red band and green band at the test and control portions of the result window indicated positive test, while the green band alone indicated absence of rotavirus antigen. Invalid result was indicated by absence of green control band or both the control band and the test band. Intensity of the red band is directly proportional to the virus concentration. Rotavirus was recovered from male calves (10/80: 12.5%) at similar rates in the female calves (5/40: 12.5%) and no statistically significant association ($\chi^2 = 0.00$, $p = 1$) existed between rotavirus infection and gender of the calves (Table 3).

3.2. Genotypes of the Detected Rotaviruses from Bovine Calves

From the 10 rotavirus positive stool samples that were submitted to genotyping assays, 14 isolates comprising 6 strains were identified (Table 4). The gel documentation of some of the electrophoretic assays for the VP7 genotyping are presented in Figure 2 and Figure 3 respectively. The full length VP7 genes (1062 bp) of strains that could not be typed by multiplex semi-nested PCR were determined by nucleotide sequencing. G-genotypes comprising G1, G5, G9, G10 and G12 were identified of which the most prevalent types were G1. The only identified P genotypes was P[6]. Multiple genotypes co-infection by G1G5 was observed in sample EK21, and G1G10 in samples OD44 and OD72 (Figure 3). The Genotype
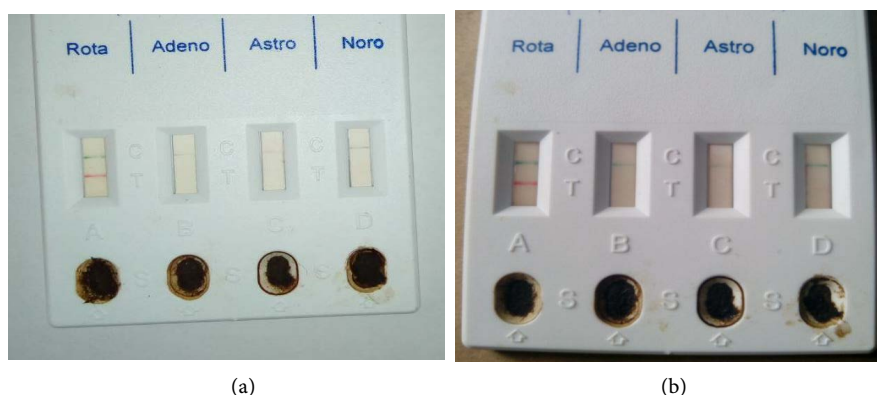
| | (a) | | | (b) |

Figure 1. (a) Viral Assay of bovine fecal sample (EK32) from Ekiti State showing positive result for Rotavirus using Certest Quadruple EIA kit; (b) Viral Assay of bovine fecal sample (OD55) from Ondo State showing positive result for Rotavirus using Certest Quadruple EIA kit.

Table 3. Rate of detection of Bovine Rotaviruses and gender of the Calves.

Gender	Rate of BoRVA detection		*p*-value
	Total No. Tested (%)	Total No. Positive (%)	
Male	80 (66.7)	10 (12.5)	1
Female	40 (33.3)	5 (12.5)	
Total	120	15 (12.5)	

BoRVA = Bovine group A Rotavirus. Chi square equals 0.00 with 1 degree of freedom. The *p*-value equals 1. No statistically significant association existed between rotavirus infection and gender of the calves.

Table 4. VP7 and VP4 genotypes and Identities of Bovine Rotavirus isolates from the positive stool samples of Calves in selected Cattle herds in Southwest, Nigeria.

| Samples	VP7 G-Typing with Primer set		VP4 P-Typing with Primer set		G&P Combinations of isolated strains	Identities of the Isolated strains
	A	B	C	D		
EK21	1062 bp = G1 780 bp = G5	0	267 bp = P[6]	0	G1P[6] G5P[6]	RVA/Bovine-wt/NGR-EK21A/2014/G1P[6] RVA/Bovine-wt/NGR-EK21B2014/G5P[6]
EK24	1062 bp = G1	0	267 bp = P[6]	0	G1P[6]	RVA/Bovine-wt/NGR-EK24/2014/G1P[6]
EK31	0	0	267 bp = P[6]	0	G9P[6]	RVA/Bovine-wt/NGR-EK31/2014/G9P[6]
EK32	1062 bp 749 bp = G1 231bp = G9		267 bp = P[6]	0	G1P[6] G9P[6]	RVA/Bovine-wt/NGR-EK32A/2014/G1P[6] RVA/Bovine-wt/NGR-EK32B/2014/G9P[6]
OD44	1062 bp = G1	276 bp = G10	267 bp = P[6]	0	G1P[6] G10P[6]	RVA/Bovine-wt/NGR-OD44A/2014/G1P[6] RVA/Bovine-wt/NGR-OD44B/2014/G10P[6]
OD55	1062 bp = G1	0	267 bp = P[6]	0	G1P[6]	RVA/Bovine-wt/NGR-OD55/2014/G1P[6]
OD65	0	394 bp	267 bp = P[6]	0	G12P[6]	RVA/Bovine-wt/NGR-OD65/2014/G12P[6]
OD72	1062 bp = G1	276 bp = G10	267 bp = P[6]	0	G1P[6] G10P[6]	RVA/Bovine-wt/NGR-OD72A/2014/G1P[6] RVA/Bovine-wt/NGR-OD72B/2014/G10P[6]
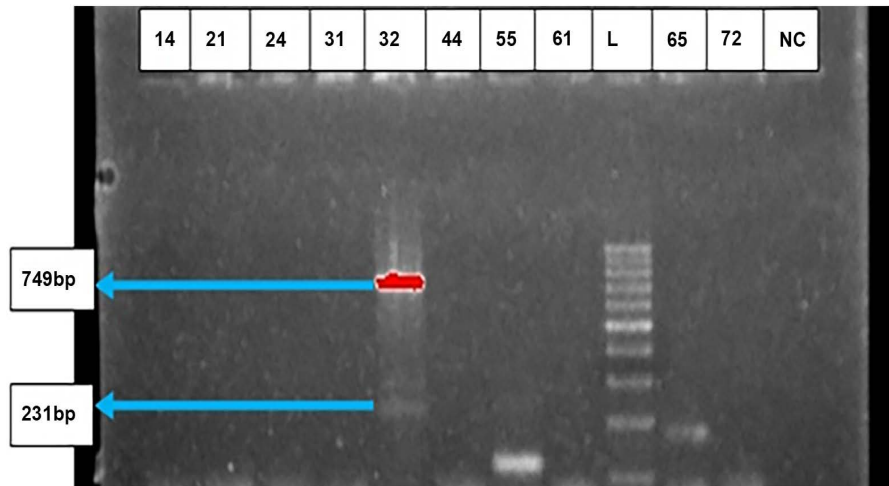
Figure 2. Gel electrophoresis of bovine rotavirus VP7 genotyping Assay showing G1 (749 bp) and G9 (231 bp) coinfection in sample EK32. L is molecular weight DNA ladder, while NC is negative control.
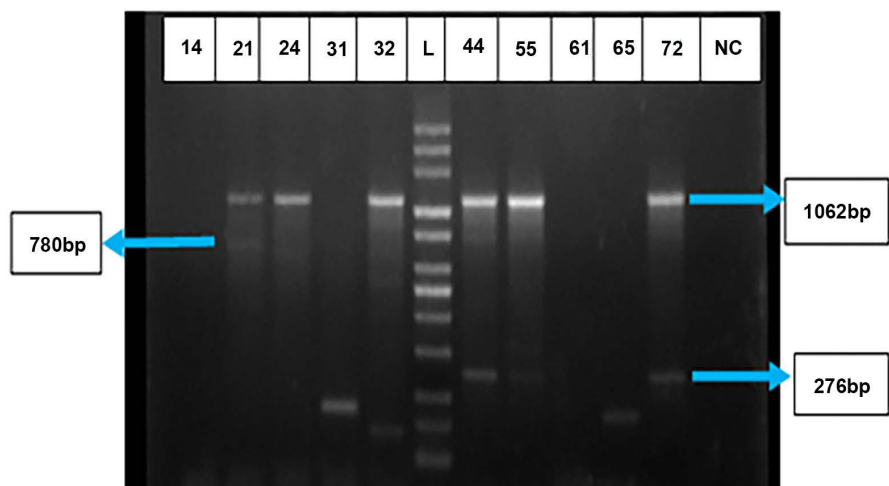

Figure 3. Gel electrophoresis of bovine rotavirus VP7 genotyping Assay showing G1 in sample EK24, EK31, EK32, OD55, and G1G10 in samples OD44 and OD72, G1 (1062 bp) and G5 (780 bp) coinfection in sample EK21.

identities of the rotavirus isolates from the positive stool samples are presented in Table 4. By genotype combinations, strain G1P[6] was the predominant strain identified, while G5P[6], G9P[6], and G10P[6] occurred at the same frequency.

3.3. Phylogenetic Relationships of Bovine Rotavirus Isolates in This Study with Other Reported Strains around the World

The bovine RVA G1 isolates showed close identity to a Nigerian human isolate detected in year 2014 while the other G1G10 mixed genotype was similar to an India isolate. The G10 genotype had 85% nucleotide similarity to a human G10 isolate detected in Honduras in year 2011 to which it clustered. The G12 isolate in this study is closely related to G12 strains identified in India in year 2009 (Figure 4).
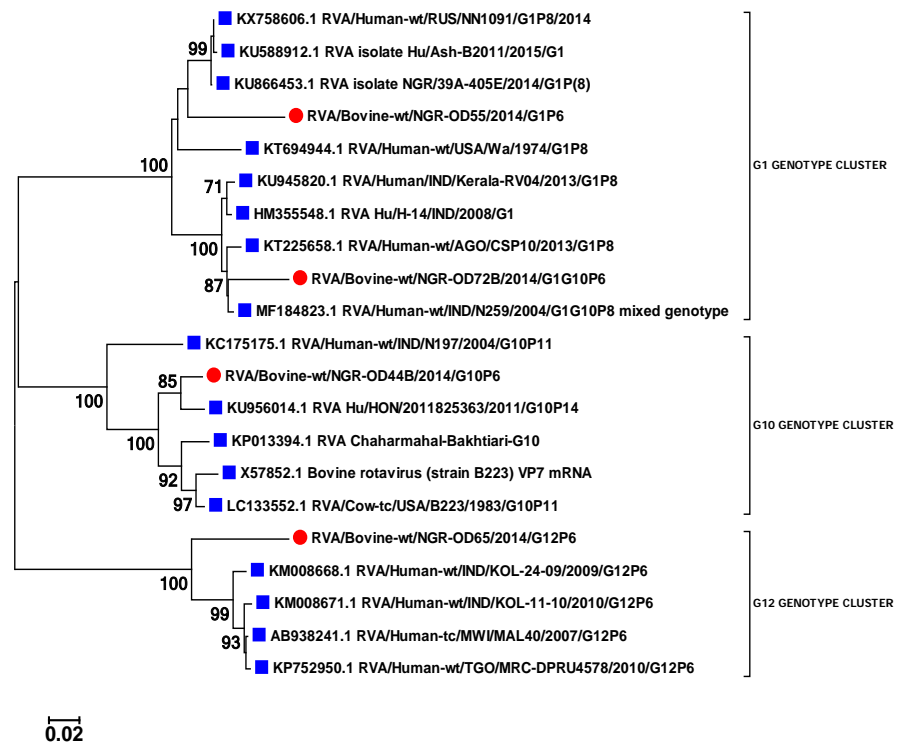
Figure 4. Phylogenetic tree of nucleotide sequences of VP7 gene of genotypes G1, G10 and G12 bovine rotavirus strains showing the genetic relationship between strains of the bovine rotaviruses from southwest Nigeria and other reference strains. The tree was elaborated by the neighbor-joining method using clustal W. Significant bootstrap values (1000 replicates) ≥ 70% supporting each node are indicated. Scale bar (0.02) indicates genetic distance expressed as number of nucleotide substitutions per site. Rotaviruses analyzed in this study are shown in red circles. References in blue squares and accession numbers for the sequences used in the gene comparisons are indicated in the phylogram.

4. Discussion

Bovine rotaviruses (BoRVAs) are important cause of neonatal calf diarrhea throughout the world and a significant cause of loss in the cattle industry [10]. In this study, EIA detected 12.5% positive bovine rotavirus cases (15/120). This rate is in line with the previous reports [19] [20]. On the molecular genotyping, 66.7% (10/15) of the EIA positive samples were successfully genotyped from which VP7 genotypes G1, G5, G9, G10, G12, and VP4 genotype P[6] were identified. The zoonotic potential of G10 genotype of bovine strain was evidenced by its transmission from cattle to humans and vice-versa as previously reported and further corroborated by the detection of the G10 strain from buffalo calves and much recently from this study in Nigeria [14] [21] [22]. The presence of rotavirus in many species of domestic animals results in serious epidemiological problems whose significance is becoming evident by the detection of genotypes of the virus believed to be strictly of animal origin in human subjects and vice-versa [23]. This is because rotavirus possesses segmented dsRNA genome with inherent capability to undergo re-assortment during co-infections resulting in progeny viruses with novel or atypical antigenic or virulent phenotypes. The close

contact between animals and humans in developing countries promote interspecies transmission and co-infections by multi serotypes. Therefore animal rotaviruses are considered as a potential threat to humans due to the possibility of genetic re-assortments. These viruses from animal origin are also known to eventually contaminate water bodies and food crops and could be transmitted by raw food materials especially vegetables, fruits and other farm produce.

Presence of several uncommon genotypes among human population similar to that detected in domestic animals suggests their potential threat for zoonotic transmission. Spread of virulent strains from animal to human is indisputable. In the past, animal strains such as the G6P[6] were detected in Belgium and Burkina Faso and reportedly linked to interspecies transmission from cattle to humans [24] [25]. One of the development from this is the formulation of a human–bovine rotavirus (116E) vaccine, ROTAVAC, recently introduced into the national immunization programme of India and reported to be effective and well tolerated in Indian infants [26]. However the detection of human strains in animal species as observed in this study is less frequently reported. The detection of this group A rotaviruses is in tandem with the report of [27] who provided evidence for the strongest identity of human and bovine strains indicating that the RVA VP7s of bovine origin are shared in strains of human rotaviruses. Although previous report of G6 animal strains evolving from G2 or G8 RVA strains with DS-1-like background, acquiring the G6 VP7 gene from a human G6P[9] RVA and not from an artiodactyl G6 RVA strain have been reported in the literature, G6 strains were not found in this study [28].

Phylogenetic analysis shows that the circulating bovine RVA G1 isolates that was detected in year 2014 showed close identity to a Nigerian human isolate detected in year 2014 while the other G1G10 mixed genotype was similar to an India isolate. It is interesting to note that the G10 genotype identified in this study had 85% nucleotide similarity to a human G10 isolate detected in Honduras in year 2011 to which it clustered. This underscores the possible transmission origin of the strain and the propensity of human-animal interspecies transmission. Furthermore, the only G12 isolate in this study is the first report of bovine G12 strains from Nigeria, closely related to G12 strains identified in India in 2009 indicating Indian lineage. This study further corroborates the zoonotic transmission between human and bovine group A rotaviruses as reported by [29] and [30] in Iraq. The worldwide predominantly encountered G and P genotype combinations responsible for 80% - 90% of global human diarrhoea disease burden, on which the Live oral Monovalent Rotarix®, the Tetravalent Rotashield® (RRV-TV), and Pentavalent RotaTeq® (RV5) Rotavirus vaccines were based are the G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] [31]. In the sub-Saharan Africa, efficacies of these licensed vaccines are challenged by mixed infections, unusual animal strains, and malnutrition. Therefore, in addition to the potential exposure of the naïve host to the new zoonotic strains, and the possible recombination and reassortment in the susceptible human, detection of this animal strains will potentially impact negatively on the efficacies of any licensed vaccines to be introduced in Nigeria.

5. Conclusion

This study contributes to surveillance for animal and human rotaviruses needed to determine the diversity of rotaviruses in various parts of Nigeria before adopting any Rotavirus National immunization programs. The results indicate that Rotaviruses were present and associated with diarrhea in freely ranged bovine calves in southwest Nigeria at a rate of 12.5%. The detected strains were identified to be group A rotavirus genotypes G1, G5, G9, G10, G12, and P[6] strains thereby indicating possible interspecies transmission from human to calves with potentials from bovine to humans and may impact negatively on potential vaccines to be introduced for vaccination efforts in Nigeria. This study reports the first detection of bovine G12 strains from Nigeria.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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