Arch Virol (2015) 160:447–451
DOI 10.1007/s00705-014-2271-4

BRIEF REPORT

Phylogenetic analysis of a G6P[5] bovine rotavirus strain isolated in a neonatal diarrhea outbreak in a beef cattle herd vaccinated with G6P[1] and G10P[11] genotypes

Thais Neris da Silva Medeiros · Elis Lorenzetti · Alice Fernandes Alfieri · Amauri Alcindo Alfieri

Received: 1 August 2014 / Accepted: 26 October 2014 / Published online: 7 November 2014

Abstract The aim of this study was to perform the molecular characterization of the eleven genes of a G6P[5] bovine group A rotavirus (RVA) strain detected in a diarrhea outbreak from a vaccinated beef cattle herd. The outbreak affected 80 % of calves between 15–30 days old. RVA was identified by RT-PCR in 12 (70.6 %) out of 17 diarrheic fecal samples evaluated. The rotavirus wild-type strain had the genotype constellation G6(IV)-P[5](IX)-I2c-R2-C2-M2-A3-N2-T6-E2e-H3a. This study confirms the importance of homotypic immunity against the bovine RVA P[5] genotype in neonatal diarrhea in cattle herds that are regularly vaccinated against rotaviruses.

Bovine group A rotavirus (RVA) is one of the main etiological agents of neonatal diarrhea in calves worldwide. Morbidity and mortality rates due to bovine RVA infections are high, which in turn cause important direct and indirect economic losses to beef and dairy production [1].

Rotavirus belongs to the family *Reoviridae*, and it is surrounded by a triple-layered protein capsid. The genome is formed by 11 double-stranded RNA segments, which encode six structural proteins (VP1-VP4, VP6, and VP7) and six non-structural proteins (NSP1-NSP5/6) [2]. Rotaviruses (RVs) are classified into eight distinct groups/species (A-H) [3, 4].

The VP7 and VP4 proteins of RVA are located in the outer layer of the capsid and induce neutralizing antibodies. The antigenic variation of the genome segments that code for these proteins determines the binary RVA genotype classification system [2]. Currently, 27 G (VP7) and 37 P genotypes (VP4) of RVA have been described in mammals and avian species [5, 6].

In 2008, the Rotavirus Classification Working Group (RCWG) defined the notations Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (x-Arabic numbers starting from 1) to VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 genes of RV strains [5]. Until now, the following genotypes have been described: VP6, I1-I17; VP1, R1-R9; VP2, C1-C9; VP3, M1-M8; NSP1, A1-A18; NSP2, N1-N10; NSP3, T1-T12; NSP4, E1-E15; NSP5/6, H1-H11 [6–10].

The most common combinations of G and P genotypes found in bovine RVA strains isolated from diarrhea episodes in calves are G6P[1] (Nebraska calf diarrhea virus [NCDV]-Lincoln), G6P[5] (UK), G8P[1] (A5), and G10P[11] (B223) [11–14]. These genotypes have been described in Brazil with frequencies of 9.7 % (3/31) to 33.3 % (12/36), 8.6 % (3/35) to 40 % (20/50), 16.7 % (6/36), and 12.9 % (4/31) to 16 % (8/50), respectively [15–17]. G6P[5] has been reported to be a common genotype in bovine RVA strains and has been found in cattle herds from Asia [18], Europe [14], Oceania [13], and America [19, 20].

The RVA G6 genotype is divided into five lineages (I–V). The bovine RVA strains can be found in the lineages G6-II, G6-III, G6-IV, and G6-V. The P[5] genotype is divided into eight (VIII) lineages. The lineages P[5]-I, P[5]-II, P[5]-III, P[5]-V, P[5]-VI, P[5]-VII, and P[5]-VIII are composed of bovine strains [21].

T. N. da Silva Medeiros · E. Lorenzetti · A. F. Alfieri · A. A. Alfieri (✉)
Laboratory of Animal Virology, Department of Veterinary Preventive Medicine, Universidade Estadual de Londrina, Celso Garcia Cid Road, PR455 Km 380, P.O. Box 10.011, Londrina, PR 86057-970, Brazil
e-mail: alfieri@uel.br

Table 1 Previously described rotavirus strains with nucleotide sequence identity to the G6P[5] Brazilian bovine RVA field strain (BRA1532)

Gene	BRA1532 genotype	Percent nucleotide sequence identity			
		NCDV	UK	WC3	Most similar strain
VP1	R2	93.3	91.3	95.7	WC3-Bo
VP2	C2	94.4	94.7	97.2	WC3-Bo
VP3	M2	94.8	94.3	93.3	NCDV-Bo
VP4	P[5]-IX	64.5	86.9	89.6	VMRI-Bo (92)
VP6	I2c	92.3	97.2	96.3	KJ9-1-Bo (97.8)
VP7	G6-IV	91.9	93.0	91.4	UK-Bo
NSP1	A3	94.8	96.9	93.3	UK-Bo
NSP2	N2	94.8	88.7	95.1	PTRV-Si (96.5)
NSP3	T6	92.6	80.5	92.6	RF-Bo (92.8)
NSP4	E2e	93.6	86.9	91.9	NCDV-Bo
NSP5/6	H3a	98.1	92.7	95.9	PTRV-Si (98.9)

Diarrhea in calves can be prevented by appropriate management practices and programs for vaccination of pregnant cows [16]. However, vaccine failures may occur for many reasons, such as reassortment between strains with different genes [22], antigenic or genetic diversity [23], interaction between different or less common genotypes [16, 24, 25], insufficient heterologous immunity [26], and inappropriate vaccination management [27].

The aim of this study was to perform a molecular characterization of the eleven genes of a G6P[5] bovine RVA strain detected in a neonatal diarrhea outbreak in a beef cattle herd that had been regularly vaccinated with G6P[1] and G10P[11] strains.

The beef cattle farm was located in Mato Grosso do Sul state, Central-West Brazil. The herd was managed on extensive pasture and had good health and nutritional practices. The farm had adopted a breeding and, consequently, birthing season of 90 days. In total, 600 cows were divided in lots ranging from 100 to 120 animals. During the neonatal diarrhea outbreak, there were 170 crossbred calves (Nelore × Angus), 15 to 30 days old.

Every cow was regularly vaccinated for neonatal diarrhea control with an inactivated commercial vaccine containing the bovine RVA genotypes G6P[1] (NCDV-Lincoln strain) and G10P[11] (B223 strain), bovine coronavirus (Hanson strain), *Clostridium perfringens* type C (toxoid/beta toxin, NL-1003 strain), and *Escherichia coli* pili adherence factor (K99 strain). The vaccination schedule was according to the manufacturer's instructions.

Diarrheic fecal samples ($n = 17$) were collected from calves aged less than 30 days old. The fecal samples were stored at −20 °C until analysis. The study was submitted to the Ethics Committee on Animal Experiments of the Universidade Estadual de Londrina and approved (46/09).

Nucleic acid was extracted using a combination of phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidinium isothiocyanate [28] extraction methods.

All diarrheic fecal samples was subjected to RT-PCR assay with previously reported consensus primers to amplify VP7 (1,062 bp) and VP4 (VP8*) (876 bp) genes to determine the G and P genotype, respectively [29–31]. A diarrheic fecal sample (BRA1532) was selected according to the quality score for analysis of the eleven genes using previously described primers [32–36].

The RT-PCR products were purified using an Illustra GFX PCR DNA and Gel Band Purification Kit and sequenced using an ABI3500 Genetic Analyzer sequencer with a BigDye® Terminator v3.1 Cycle Sequencing Kit.

Nucleotide quality analysis and contig assembly of the RVA gene sequences were performed with Phred and CAP3 software, respectively. The identity matrix was constructed using BioEdit software version 7.0.8.0. Phylogenetic trees based on the nucleotides were obtained using the neighbor-joining method in MEGA v6 software. The bootstrapping probabilities were calculated using 1,000 replicates. The sequences described in this study are available in GenBank with the accession numbers JQ943546 to JQ943578.

Eighty percent (136/170) of the calves that were 15 to 30 days old had diarrhea. All diarrheic calves were treated with broad-spectrum antibiotics by the parenteral route but were unresponsive to antibiotic therapy, and 2.94 % (4/136) of the calves died.

Before analysis for bovine RVA, the diarrheic fecal samples were evaluated by the modified Ziehl-Neelsen technique [37] and SN-PCR assay [38] for *Cryptosporidium* spp. and bovine coronavirus detection, respectively, and the results were negative (data not shown).

RT-PCR assays using consensus primers of VP7 and VP4 (VP8*) genes showed that 12 (70.6 %) out of 17 diarrheic fecal samples analyzed were RVA positive. The nucleotide sequence analysis of the VP7 and VP4 (VP8*) genes of 12 Brazilian wild-type bovine RVA strains showed the highest nucleotide sequence identity (94.9 %) to G6 genotype lineage IV (OH-4 equine strain) (Fig. 1a), and 92 % with the bovine strain VMRI, which belongs to the P[5] genotype lineage VIII (Fig. 1b). The cutoff value to be considered of the same lineage of the P[5] genotype is 96 % nucleotide sequence identity [21]; therefore, we propose a new lineage, named P[5]-IX. In the phylogenetic tree, the BRA1532 strain formed a new branch separated from the other lineages of the P[5] genotype.

For the VP6, VP1-VP3, and NSP1-NSP5/6 genes, the nucleotide sequence analysis of the RT-PCR products of the bovine RVA BRA1532 field strain revealed the

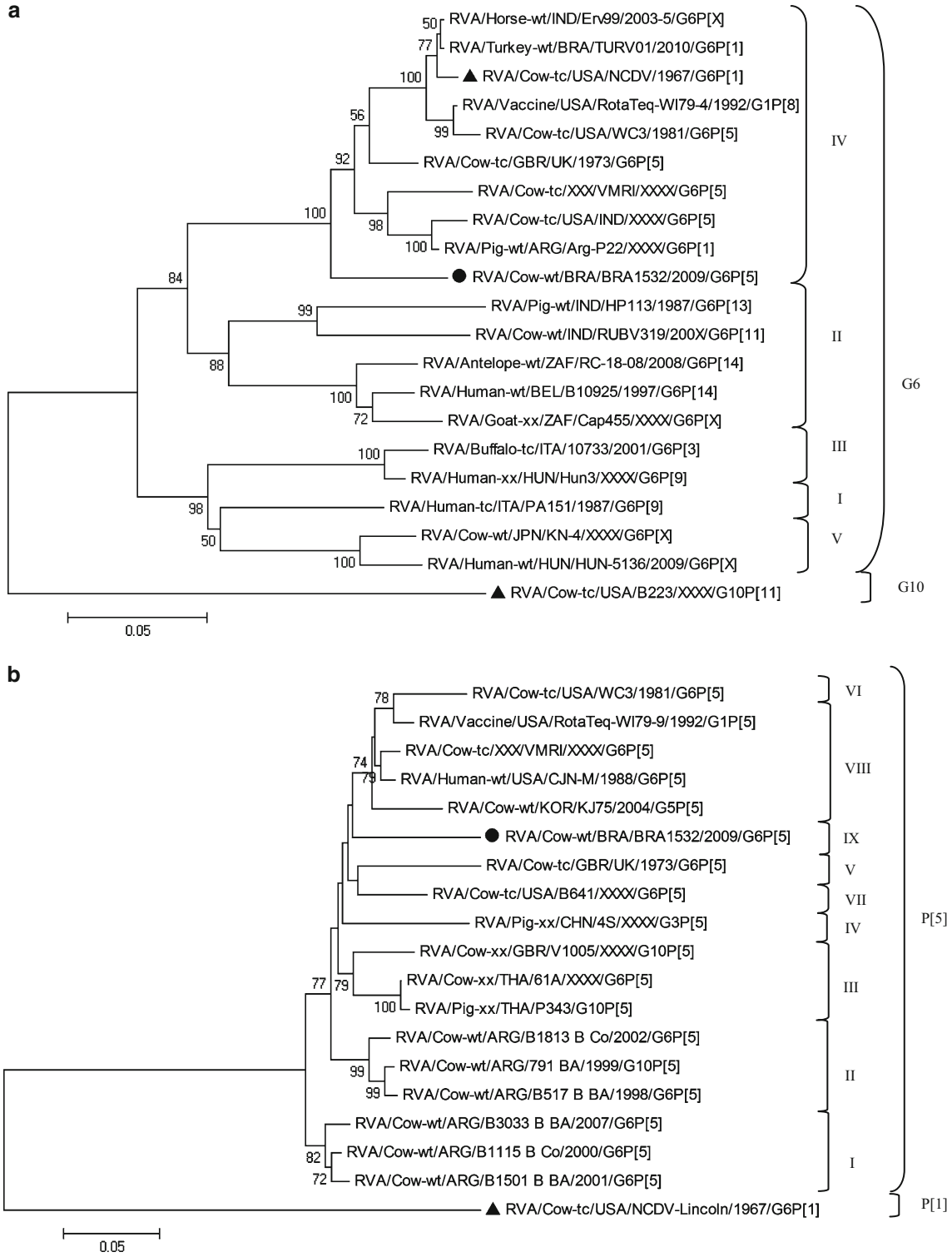

Fig. 1 **a.** Phylogenetic tree of the VP7 gene, reconstructed using the sequence of a 933-bp amplicon (nt 64–996) of genotype G6 of the BRA1532 strain, represented by a filled circle. The vaccine strains are indicated by filled triangles. The numbers adjacent to the nodes represent the percentage of bootstrap support (1,000 replicates) for the clusters. Bootstrap values less than 50 % are not shown. **b.** Phylogenetic tree of the VP4 (VP8*) gene, reconstructed using the sequence of a 644-bp amplicon (nt 61–704) of genotype P[5] of the BRA1532 strain, represented by a filled circle. The vaccine strain is indicated by a filled triangle. The numbers adjacent to the nodes represent the percentage of bootstrap support (1,000 replicates) for the clusters. Bootstrap values less than 50 % are not shown

presence of the following genotypes: G6(IV)-P[5](IX)-I2c-R2-C2-M2-A3-N2-T6-E2e-H3a. The bovine WC3 strain used in the human vaccine and the BRA1532 strain has the same constellation of genotypes. However, the Brazilian field strain showed high nucleotide sequence identity to the WC3 strain only in the VP1 and VP2 genes (Table 1).

The 70.6 % (12/17) of diarrheic fecal samples that were positive for bovine RVA in this study suggested that rotavirus was the etiological agent involved in this neonatal diarrhea outbreak. Additionally, all samples were negative for common enteropathogens such as bovine coronavirus and *Cryptosporidium* spp. (data not shown), and the diarrheic calves were unresponsive to two or three doses of broad-spectrum antibiotic therapy [39].

The nucleotide sequence analysis of the VP1–VP3, VP4, VP6–VP7, and NSP1-NSP5/6 genes of the BRA1532 strain showed the same genome constellation described in the bovine WC3 (G6P[5]) strain; however, the Brazilian bovine RVA strain belonged to the P[5] genotype lineage IX, and the WC3 strain belonged to the P[5] genotype lineage IV. The BRA1532 strain displayed the T6 (NSP3 gene) genotype, which was distinct from the gene found in the bovine UK strain (G6P[5]), which belongs to the T7 genotype. Additionally, the VP4 (VP8*) and NSP5/6 genes of the BRA1532 strain belonged to different lineages (P[5]-IX and H3a) when compared with the UK strain (P[5]-V and H3e) [6, 40]. According to the NCDV-Lincoln strain, the field Brazilian strain shared the same genotypes and lineages for all genes analyzed, which are present in the commercial vaccine used in this beef cattle herd, with the exception of the VP4 (VP8*) gene.

There is significant homotypic divergence within genotype G6 of RVA strains identified in human and bovine hosts, suggesting the existence of antigenic differences between G6 strains [41, 42]. Homologous immunity occurs within the same G6 genotype, and even a small genotypic difference is enough to result in insufficient protection [26].

Some studies have shown that vaccination with the prototype G6P[1] of bovine RVA results in poor heterologous protection against RVA G6 strains containing different P genotypes from the vaccine [27, 43]. Moreover, commercial vaccines containing the genotype G6P[1] may not be as effective, as P[1] may not be the most common genotype depending on the geographic region studied [44]. The vaccine pressure may generate competition, selection or variation between strains [45], which could be responsible for the introduction or emergence of novel adapted strains or differences in genotypes [23, 45].

Different genotypes have caused diarrhea in calves born to dams vaccinated against rotavirus due to poor heterologous protection against a reassortment between B641 (G6P[5]) and B223 (G10P[11]) bovine RVA strains [24]. However, it has been reported that immunity against the different G6 strains is due, at least in part, to heterologous protection against another lineage, even when the P genotype is different [41].

A polyvalent vaccine may not offer protection against all of the currently circulating bovine RVA genotypes, and multiple G and P genotypes can circulate simultaneously in a given population. Their impact on morbidity and mortality rates, high treatment costs, and reduced growth rates would not be minimized [45].

Monitoring the G and P genotypes of bovine RVA strains that are circulating or involved in outbreaks of neonatal calf diarrhea in a vaccinated cattle herd should be performed more often for a comprehensive analysis of the genetic diversity of RVA. It should also be emphasized that a new classification system would also help in understanding the inefficient protection.

In conclusion, the G6-IV and P[5]-IX genotypes were identified in the bovine RVA field strain as the cause of a diarrhea outbreak in a vaccinated beef cattle herd. These genotypes were distinct from the G and P genotypes of bovine RVA strains included in the commercial vaccine used in the herd. These results highlight the importance of homotypic immunity and monitoring of the bovine RVA genotypes circulating in cattle populations.

Acknowledgments This study was supported by the following Brazilian institutions: National Council of Scientific and Technological Development (CNPq), Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES), Financing of Studies and Projects (FINEP), and the Araucária Foundation (FAP/PR). Alfieri, A.A., Alfieri, A.F. and Lorenzetti, E. are recipients of CNPq fellowships.

References

1. Holland RE (1990) Some infectious causes of diarrhea in young farm animals. Clin Microbiol Rev 3:345–375
2. Estes MK, Kapikian AZ (2007) Rotaviruses. In: Knipe DM, Howley PM (eds) Fields virology, 5th edn. Lippincott Williams & Wilkins, Philadelphia, pp 1918–1974
3. Attoui H, Mertens PPC, Becnel J et al (2012) Family: Reoviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ (eds) Virus taxonomy: ninth report of the ICTV. Elsevier Academic Press, Amsterdam, pp 541–637
4. Matthijnssens J, Otto PH, Ciarlet M et al (2012) VP6-sequence-based cutoff values as a criterion for rotavirus species demarcation. Arch Virol 157:1177–1182
5. Matthijnssens J, Ciarlet M, Rahman M et al (2008) Recommendations for the classification of group A rotaviruses using all 11 genomic RNA segments. Arch Virol 153:1621–1629
6. Matthijnssens J, Ciarlet M, McDonald SM et al (2011) Uniformity of rotavirus strain nomenclature proposed by the rotavirus classification working group (RCWG). Arch Virol 156: 1397–1413
7. Trojnar E, Sachsenroder J, Twardziok S et al (2013) Identification of an avian group A rotavirus containing a novel VP4 gene of

close relationship to those of mammalian rotaviruses. J Gen Virol 94(Pt1):136–142
8. Guo D, Liu J, Lu Y et al (2012) Full genomic analysis of rabbit rotavirus G3P[14] strain N5 in China: identification of a novel VP6 genotype. Infect Genet Evol 12:1567–1576
9. Papp H, Al-Mutairi LZ, Chehadeh W et al (2012) Novel NSP4 genotype in a Camel G10P[15] rotavirus strain. Acta Microbiol Immunol Hung 59:411–421
10. Jere KC, Esona MD, Ali YH et al (2014) Novel NSP1 genotype characterized in an African camel G8P[11] rotavirus strain. Infect Genet Evol 21:58–66
11. Gulati BR, Nakagomi O, Koshimura Y et al (1999) Relative frequencies of G and P types among rotaviruses from Indian diarrheic cow and buffalo calves. J Clin Microbiol 37:2074–2076
12. Fukai K, Maeda Y, Fujimoto K et al (2002) Changes in the prevalence of rotavirus G and P types in diarrheic calves from the Kagoshima prefecture in Japan. Vet Microbiol 86:343–349
13. Swiatek DL, Palombo EA, Lee A et al (2010) Detection and analysis of bovine rotavirus strains circulating in Australian calves during 2004 and 2005. Vet Microbiol 140:56–62
14. Midgley SE, Bányai K, Buesa J et al (2012) Diversity and zoonotic potential of rotaviruses in swine and cattle across Europe. Vet Microbiol 156:238–245
15. Alfieri AF, Barreiros MAB, Leite JPG et al (2004) G and P genotypes of group A rotavirus strains circulating in calves in Brazil, 1996–1999. Vet Microbiol 99:167–173
16. Barreiros MAB, Alfieri AF, Médici KC et al (2004) G and P genotypes of group A rotavirus from diarrhoeic calves born to cows vaccinated against the NCDV (P[1], G6) rotavirus strain. J Vet Med B Infect Dis Vet Public Health 51:104–109
17. Caruzo TA, Brito WM, Munford V et al (2010) Molecular characterization of G and P-types bovine rotavirus strains from Goiás, Brazil: high frequency of mixed P-type infections. Mem Inst Oswaldo Cruz 105:1040–1043
18. Ishizaki H, Sakai T, Sharahata T et al (1996) The distribution of G and P types within isolates of bovine rotavirus in Japan. Vet Microbiol 48:367–372
19. Garaicoechea L, Bok K, Jones LR et al (2006) Molecular characterization of bovine rotavirus circulating in beef and dairy herds in Argentina during a 10-year period (1994–2003). Vet Microbiol 118:1–11
20. Freitas PPS, Uyemura SA, Silva DG et al (2011) Rotavirus in cattle: risk factors, prevalence and antigenic characterization from dairy calves samples in São Paulo State, Brazil. Arq Bras Med Vet Zootec 63:820–827
21. Badaracco A, Garaicoechea L, Matthijnssens J et al (2013) Phylogenetic analysis of typical bovine rotavirus genotypes G6, G10, P[5] and P[11] circulating in Argentinean beef and dairy herds. Infect Genet Evol 18:18–30
22. Martella V, Bányai K, Matthijnssens J et al (2010) Zoonotic aspects of rotaviruses. Vet Microbiol 140:246–255
23. Phan TG, Khamrin P, Quang TD et al (2007) Detection and genetic characterization of group A rotavirus strains circulating among children with acute gastroenteritis in Japan. J Virol 81:4645–4653
24. Lu W, Duhamel GE, David BA et al (1994) Serological and genotypic characterization of group A rotavirus reassortants from diarrheic calves born to dams vaccinated against rotavirus. Vet Microbiol 42:159–170
25. Rodríguez-Limas WA, Flores-Samaniego B, de la Mora G et al (2009) Genotypification of bovine group A rotavirus in México. Vaccine 27:6411–6414
26. Woode GN, Kelso NE, Simpson TF et al (1983) Antigenic relationships among some bovine rotaviruses: serum neutralization and cross-protection in gnotobiotic calves. J Clin Microbiol 18:358–364
27. Alkan F, Ozkul A, Oguzoglu TC et al (2010) Distribution of G (VP7) and P (VP4) genotypes of group A bovine rotaviruses from Turkish calves with diarrhea, 1997–2008. Vet Microbiol 141:231–237
28. Alfieri AA, Parazzi ME, Takiuchi E et al (2006) Frequency of group A rotavirus in diarrhoeic calves in Brazilian cattle herds, 1998–2002. Trop Anim Health Prod 38:521–526
29. Gouvea V, Glass RI, Woods P et al (1990) Polymerase Chain Reaction amplification and typing of rotavirus nucleic acid from stool specimens. J Clin Microbiol 28:276–282
30. Gentsch JR, Glass RI, Woods P et al (1992) Identification of group A rotavirus gene 4 types by polymerase chain reaction. J Clin Microbiol 30:1365–1373
31. Martella V, Ciarlet M, Bányai K et al (2006) Identification of a novel VP4 genotype carried by a serotype G5 porcine rotavirus strain. Virology 346:301–311
32. Varghese V, Ghosh S, Das S et al (2006) Characterization of VP1, VP2 and VP3 gene segments of a human rotavirus closely related to porcine strains. Virus Genes 32:241–247
33. Iturriza-Gómara M, Wong C, Blome S et al (2002) Molecular characterization of VP6 genes of human rotavirus isolates: correlation of genogroups with subgroups and evidence of independent segregation. J Virol 76:6596–6601
34. Matthijnssens J, Ciarlet M, Heiman E et al (2008) Full genome-based classification of rotaviruses reveals a common origin between human Wa-like and porcine rotavirus strains and human DS-1-like and bovine rotavirus strains. J Virol 82:3204–3219
35. Lee C-N, Wang Y-L, Kao C-L et al (2000) NSP4 gene analysis of rotaviruses recovered from infected children with and without diarrhea. J Clin Microbiol 38:4471–4477
36. Mohan KVK, Atreya CD (2001) Nucleotide sequence analysis of rotavirus gene 11 from two tissue culture-adapted ATCC strains, RRV and Wa. Virus Genes 23:321–329
37. Henriksen A, Pohlenz JFL (1981) Staining of *Cryptosporidium* by a modified Ziehl-Nielsen technique. Acta Vet Scand 22:594–596
38. Takiuchi E, Stipp DT, Alfieri AF et al (2006) Improved detection of bovine coronavirus N gene in faeces of calves infected naturally by a semi-nested PCR assay and an internal control. J Virol Methods 131:148–154
39. Bendali F, Sanaa M, Bichet H et al (1999) Risk factors associated with diarrhoea in newborn calves. Vet Res 30:509–522
40. De Grazia S, Martella V, Rotolo V et al (2011) Molecular characterization of genotype G6 human rotavirus strains detected in Italy from 1986 to 2009. Infect Genet Evol 11:1449–1455
41. Chang KO, Parwani AV, Saif LJ (2000) Comparative sequence analysis of the VP7 genes of G6, G8 and G10 bovine group A rotarviruses and further characterization of G6 subtypes. Arch Virol 145:725–737
42. Cooney MA, Gorrell RJ, Palombo EA (2001) Characterization and phylogenetic analysis of the VP7 proteins of serotype G6 and G8 human rotaviruses. J Med Microbiol 50:462–467
43. Clark KJ, Tamborello TJ, Xu Z et al (1996) An unusual group-A rotavirus associated with an epidemic of diarrhea among three-month-old calves. J Am Vet Med Assoc 208:552–554
44. Brito WMED, Munford V, Villaça AM et al (2000) Characterization of mixed infections with different strains of bovine rotavirus in an outbreak of diarrhea in dairy herds in Goiás, Brazil. Braz J Microbiol 31:140–145
45. Cashman O, Lennon G, Sleator RD et al (2010) Changing profile of the bovine rotavirus G6 population in the south of Ireland from 2002 to 2009. Vet Microbiol 146:238–244