Research Article

Molecular Characterization of the Porcine Group A Rotavirus NSP2 and NSP5/6 Genes from São Paulo State, Brazil, in 2011/12

Bruna Rocha Passos Barbosa, Nara Thiers Cacciatori Galleti Bernardes, Laila Andreia Rodrigues Beserra, and Fábio Gregori

Department of Preventive Veterinary Medicine and Animal Health, Faculty of Veterinary Medicine, University of São Paulo, Avenida Prof. Dr. Orlando Marques de Paiva 87, Cidade Universitária, 05508-270 São Paulo, SP, Brazil

Correspondence should be addressed to Bruna Rocha Passos Barbosa; bruna888@hotmail.com

Received 15 May 2013; Accepted 29 June 2013

Academic Editors: C. DebRoy, D. Endoh, and B. I. Yoon

Copyright © 2013 Bruna Rocha Passos Barbosa et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Rotaviruses are responsible for the acute diarrhea in various mammalian and avian species. The nonstructural proteins NSP2 and NSP5 are involved in the rotavirus replication and the formation of viroplasm, cytoplasmic inclusion bodies within which new viral particles morphogenesis and viral RNA replication occur. There are few studies on the genetic diversity of those proteins; thus this study aims at characterizing the diversity of rotavirus based on NSP2 and NSP5 genes in rotaviruses circulating in Brazilian pig farms. For this purpose, 63 fecal samples from pig farms located in six different cities in the São Paulo State, Brazil, were screened by nested RT-PCR. Seven strains had the partial nucleotide sequencing for NSP2, whereas in six, the total sequencing for NSP5. All were characterized as genotype H1 and N1. The nucleotide identity of NSP2 genes ranged from 100% to 86.4% and the amino acid identity from 100% to 91.5%. For NSP5, the nucleotide identity was from 100% to 95.1% and the amino acid identity from 100% to 97.4%. It is concluded that the genotypes of the strains circulating in the region of study are in agreement with those reported in the literature for swine and that there is the possibility of interaction between human and animal rotaviruses.

1. Introduction

Group A rotaviruses (GARV) are the major causes of viral diarrhea in a variety of animal young species worldwide [1]. In pig farms, they are responsible for economic losses due to death of animals, poor growth performance, and costs of diagnostic and treatment [2, 3]. Porcine GARVs are associated with weaning and postweaning enteritis in piglets and more often detected in piglets between 1 and 8 weeks of age [4, 5].

Rotavirus belongs to the family Reoviridae, subfamily Sedoreovirinae, and its genome consists of 11 segments of double-stranded RNA (dsRNA), which encode six structural proteins (VP1, VP2, VP3, VP4, VP6, and VP7) and six nonstructural proteins (NSP1–NSP6), with all genes being monocistronic except for segment 11 which encodes two proteins, NSP5 and NSP6 [6].

Encoded by segment 8 (1059 bp), NSP2 is a 35 kDa protein which assembles into octamers and is composed of 317 amino acids [7]. NSP2 has a role in packaging and replication, performing activities of nonspecific binding to single-stranded RNA (ssRNA) to start the synthesis of dsRNA, translocation of viral RNA during packaging, and helicase ATP-independent, triphosphatase, and NDP kinase activities [7–10].

NSP5 encoded by segment 11 (667 bp) is a 21 kDa protein that is hyperphosphorylated and O-glycosylated, and it consists of 198 amino acids, with abundance of serine (21%) and threonine (4.5%) [11–13]. NSP5 interacts with NSP2 to form cytoplasmic structures known as viroplasms, inside of which RNA replication and morphogenesis of new viral particles occur [1, 14, 15]. The interaction with NSP2 also enhances the process of NSP5 hyperphosphorylation [9, 16]. The segment 11 also contains the coding sequence of NSP6 in a second ORF, whose function is still unknown, but it preliminarily interacts with NSP5 in dimerization and hyperphosphorylation processes. However, given the low levels of expression of
NSP6, this suggests that this protein did not have an essential regulatory role [17–19].

Traditionally, GARVs are classified on the basis of two outer capsid proteins (VP4 and VP7), which induce the formation of neutralizing antibodies, with the VP7 determining the genotype G, whereas VP4 determines P genotype [1, 6]. However, a new classification for rotavirus was recently proposed which takes into account all 11 genomic segments, and the genotype N was determined for NSP2 protein and genotype H for NSP5 [20]. So far, nine genotypes N (N1 to N9) and 11 genotypes H (H1 to H11) have been identified. In humans, genotypes N1, N2, and N3 and H1, H2, and H3 have been described, whereas in pigs, H1 and N1 have been identified [5, 20]. As observed for VP4 and VP7, NSP2 and NSP5 also share genotypes between humans and animals, as previously reported by Martella et al., Matthijnssens et al., Tsugawa and Hoshino, and Khamrin et al. [5, 21–23].

The zoonotic aspect and interspecies transmission of rotavirus have been described by several authors [5, 21, 24, 25], and in this context the swines are considered as a major source of infection and important reservoirs for genetic and antigenic diversity of human rotaviruses [26, 27].

Despite the importance of genetic characterization of rotavirus circulating strains, data on the NSP2/5/6 molecular characterization in pigs in Brazil are scarce. Therefore, the objective of this study was to sequence the NSP2 and NSP5/6 genes of group A porcine rotavirus isolated from different pig herds in the São Paulo State, Brazil, in order to understand the phylogenetic relationships among them, as well as regarding other strains previously isolated from several regions around the world.

2. Materials and Methods

2.1. Samples. A total of 63 fecal samples from 34-day-old piglets with and without symptoms of diarrhea were used to study the NSP2 and NSP5 genes of GARV. The samples were collected in pig farms located in six different cities in the state of São Paulo, Brazil, from 2011 to 2012.

The rotavirus strain NCDV was used as positive control and ultrapure water previously treated with 0.1% diethyl pyrocarbonate (DEPC water) (Invitrogen) as negative control.

2.2. Detection of Rotavirus. For initial screening, the viral RNA was extracted from fecal samples using the TRIzol Reagent method (Invitrogen) according to the manufacturer’s instructions, followed by nested RT-PCR technique as described by Salem et al. [28]. The extracted RNA was denatured at 95°C for 5 minutes and then subjected to RT-PCR.

2.3. RT-PCR. The reverse transcription reaction (cDNA synthesis) was performed using the following protocol. Briefly, 7 μL of extracted RNA was added to a mixture containing 1x First Strand Buffer (Invitrogen), dNTP (1 mM each), 10 mM DTT, 1 mM Random Hexamers (50 ng/μL, Invitrogen), 200 U MMLV Reverse Transcriptase (Invitrogen), and ultrapure DEPC-treated water for a final reaction volume of 20 μL. The RT was performed at 37°C for 1 h, followed by incubation at 70°C for 15 min.

The amplification of the NSP2 partial gene (975 bp) was performed using four primers, one of them described by Matthijnssens et al. [21] and others developed in the present study based on alignments of sequences retrieved from GenBank (Table 1). For NSP5/6, the complete segment of 667 bp was amplified using the primers previously described by Matthijnssens et al. [21] (Table 1).

Thus, the PCR reaction consisted of adding 5 μL of cDNA to a mixture containing 1x PCR Buffer (Invitrogen), dNTP (0.2 mM each), 0.2 μM of each primer (Table 1), 2 mM MgCl₂, 5 U of Taq DNA Polymerase (Invitrogen), and ultrapure DEPC-treated water for a final reaction volume of 25 μL. The PCR was performed at 94°C/3 min, followed by 40 amplification cycles (94°C/45 s, 54°C/45 s, 72°C/2,5 min) and a final extension of 70°C for 7 min.

2.4. DNA Sequencing. The amplicons were purified with ExoSAP-IT (Affymetrix) according to the manufacturer’s instructions and sequenced in both directions using the BigDye Terminator Cycle Sequencing Kit v. 3.1 (Life Technologies) followed by the application of the BigDye Terminator kit (Life Technologies) for the removal of unincorporated DNA terminators, also according to the manufacturer’s instructions, on an automated DNA sequencer ABI-3500 (Applied Biosystems).

2.5. Phylogenetic Analysis. The sequences obtained were edited using Bioedit v. 7.1.3.0 program [29], and multiple alignments were done using the Clustal W v. 2.1 software [30]. The nucleotide and deduced amino acid sequences of NSP2 and NSP5/6 genes were compared with those of reference strains available at NCBI (National Center for Biotechnology Information) GenBank database using the BLAST (Basic Local Alignment Search Tool) server [31].

| Gene | Primer | Primer sequence (5’ → 3’ ) | Amplicon (bp) | Reference |
|------|--------|-----------------------------|--------------|-----------|
| NSP2 | GEN-NSP2F | GGC TT T T A A A A G C G T C T C AG | 728 | [21] |
| NSP2-RW707 | GTACCATTTCCAGTGATRTCTC | Developed in this study |
| NSP2-FW707 | CATGGTAAAGGTCACTAYAGAG | 247 |
| NSP2-RW975 | ACAGTTGACTATCTTTYTACCT | Developed in this study |
| NSP5 | GEN-NSP5F | GGCTTTTTAAGCGCTACAG | 667 | [21] |
| GEN-NSP5R | GGTCAACAAAAACGGGAGT | | | |
Phylogenetic analyses were conducted using MEGA version 5.1 [32], and the phylogenetic tree was built using the neighbor-joining method (1,000 bootstrap trials) with maximum composite likelihood as substitution model.

2.6. Nucleotide Sequence Accession Numbers. The nucleotide sequences of NSP2 and NSP5/6 genes of the rotavirus strains from this study have been deposited in GenBank under the accession numbers KC117140 to KC117146 for NSP2 and KC117147 to KC117152 for NSP5.

3. Results

3.1. Detection of Rotavirus. Seventeen of the 63 fecal samples (17/63 or 27%) tested positive for rotaviruses by nested RT-PCR [28] from five different cities (5/6).

3.2. Analysis of NSP2 Gene Sequences. We sequenced a partial NSP2 fragment (997 bp or 97%) in 7 of the 17 positive strains analyzed (SWRV1 to SWRV7).

Comparing the nucleotide and predicted amino acid sequences of the strains of the present study with others already deposited in GenBank, the data revealed that SWRV 1, 2, 6, and 7 were more closely related to the porcine rotavirus strain, YM, in terms of nucleotides (94.7–95.4%) and to bovine strains, KF44 and KF75, in terms of amino acid (98.0–98.3%). With regard to the strains SWRV 3, 4, and 5, they shared maximum identities with the human rotavirus strain, IAL28 (94.0–95.1%) in terms of nucleotide and 96.1% for amino acids. The phylogenetic tree revealed that the strains clustered together with the N1 genotype (Figure 1).

The motifs related to the functions of translocation of RNA (75 bases corresponding to the 5’ noncoding region 3’ and the beginning of ORF), to the NTPase (histidine at position 225), and to the cysteine residues 6, 8, 85, and 285, which are involved in the formation of disulfide bridges [10, 33, 34], were conserved between the samples of this study.

There was variability in the RNA-binding domain of NSP2 corresponding to amino acid residues 205–241 (A207S, R215K, I218V, and S216F, and I218A) [10, 33].

3.3. Analysis of NSP5/6 Gene Sequences. Complete sequences of these genes and their corresponding proteins were obtained in 6 of the 17 positive strains analyzed (6/17) (SWRV1 to SWRV6).

The comparison among the nucleotide and putative amino acids sequences of the strains of this study with others from human and animal previously deposited in GenBank revealed a high identity among SWRV 1, 2, and 3 and porcine strains Gottfried, RU172 and CMP034 (94.0–95.1% nucleotide and 96.1% amino acid identity). With respect to SWRV 4, 5, and 6, they also shared maximum identities with the porcine rotavirus strains, Gottfried and CMP034 (97.1%, in terms of nucleotide and 99.4% for amino acids). The phylogenetic tree revealed that the strains clustered together with the H1 genotype (Figure 2).

For NSP6, in terms of amino acids, the strains SWRV 1, 2, and 3 shared maximum identities values ranging from 95.6% with human strains 2007744509, 2008747100, and 2007719698, while SWRV 4, 5, and 6 were most closely related to porcine strain, PRG942 (98.9–100%).

The nine phosphorylation sites of NSP5 concerning serine residues at positions 2, 4, 30, 37, 42, 56, 67, 101, 127, and 163 [35] present conserved between the samples of this study, except for the residue at position 37 (S37N).

4. Discussion

Studies carried out about the occurrence of RVA in pigs from different Brazilian regions have shown a relatively wide distribution of this agent in the creations around the country [36–38]. Five of the six cities studied presented samples positive for rotavirus at an overall prevalence of 27% (17/63), very similar to that found by Gregori et al. [36] of 29.9%.

Our study revealed the circulation of genotype N1 of NSP2 in the cities sampled, which, for pigs, is the only N genotype reported so far, but that has also been described in humans, cattle, and cats [5, 20, 39]. Likewise, the genotype H1 detected for NSP5 has been reported in pigs, which also occurs in humans and animals [5, 20]. It should be noted that no genotypic classification was defined for NSP6, since this protein ORF is entirely contained within the NSP5 ORF. Therefore, as suggested by Matthijnssens et al. [39], the phylogenetic analysis of both would be nearly identical.

The NSP2 genes for the strains SWRV 3, 4, and 5 were the closest related to that of the IAL28 strain, a G5 human rotavirus strain isolated in Brazil in 1992 from a child with diarrhea, and that G5 is another genotype commonly described in pigs [40]. Although the highest nucleotide and amino acid identity values for NSP5 had been with porcine rotavirus strains, phylogenetic tree deduced from this gene revealed that the strains SWRV 1, 2, and 3 segregated in a cluster that also included the human strain IAL28 with which those strains showed high nucleotide and amino acid identities ranging from 94.1 to 94.6% and 94.3%, respectively (Figure 2). The findings of genetic relationships of the porcine strains of this study with those of human rotavirus, as well as detection of genotypes common to both species, suggest the occurrence of interspecies transmission, in which the pigs likely act as a source of infection for humans [24, 39].

Several studies corroborated our findings by reporting high degrees of genetic similarity between human and animal strains, as well as the occurrence of N1 and H1 genotypes in human strains (5, 21, 27, 41). Esona et al. showed that in six African strains, the genes VP3, NSP2, and NSP5 had high identity with animal strains (83–99% and 97–99% nucleotide and amino acid values, resp.) [41]. Additionally, Ghosh et al. determined the nucleotide sequence of the genes encoding VP1, VP2, VP3, and NSP1, NSP2, and NSP3 of the porcine strain, RU172, being that NSP2 clustered in a group predominantly consisting of human rotavirus [42].

It has been shown that NSP2 motif related to the RNA binding consists of the amino acid residues 205–241, while the cysteine residues 6, 8, 85, and 285 are involved in disulfide bridges which ensure the octameric structure of this protein [10, 33]. Furthermore, these studies also reveal an extensive
Figure 1: Phylogenetic tree constructed with the neighbor-joining method using maximum composite likelihood as substitution model for a partial region (975 bp) of the NSP2 gene of the group A porcine rotavirus of the strains SWRV1 and SWRV7 with others representing human and animal GARVs. The scale represents the number of substitutions per site. Only bootstrap values > 70% are shown. Pig strains defined in the present study are preceded by black triangles, and they are identified as accession number/host/strain/NSP2 genotype.

Conserved region of 75 bases (b) including 35b in the 5' noncoding region 3' and the first 30b of the ORF as a region in which the conformational NSP2 changes occur, leading to translocation of ssRNA. The NTPase activity of NSP2 is related to amino acid histidine at position 225, which acts as a catalyst residue for enzymatic function of this protein, so that the mutation in this residue prevents the synthesis of dsRNA [34]. Thus, mutations in regions concerning the binding domain of NSP2 with the RNA, R215K, S216F, and I218V, found in the strains of the present study in relation to the standard strain Gottfried (Accession number GU199489), have been reported in samples standard OSU, UK, NCDV,
Wa, and SA11 [33, 43, 44]. On the other hand, for mutations at positions I218A and A207S, there are no reports in the literature.

In regard to NSP5, nine phosphorylation sites were identified in this protein concerning serine residues at positions 2, 4, 30, 37, 42, 56, 67, 101, 127, and 163 [35]. Compared to the standard strain Gottfried (Accession number GU199491), the strains of this study showed a mutation from serine to asparagine at position 37 which does not seem to be significant since some isoforms of this protein may not provide the phosphorylation sites at residues 30 and/or 37, and then, serine would be absent at these sites, but without altering the phosphorylation process, as demonstrated by Sotelo et al. [35].
The data showed low rates of genetic variability for NSP2 and NSP5/6 in the strains of the present study. Indeed, these proteins are generally more stable because of structural and functional constraints and lower exposure to immune selection [45].

More genetic characterization studies would be helpful for detection of interspecies transmission [21, 27, 46]; however, only collection and analysis of RV specimens collected in close geographic and temporal proximity in a prospective manner, as shown by Steyer et al. [25], will provide firm evidence. Thus, considering that there are few studies in Brazil aiming at molecular characterization of NSP2 and NSP5 in pigs, it becomes imminent monitoring swine herds and functional constraints and lower exposure to immune selection [45].

5. Conclusion

The molecular analyses performed in this study indicated that most of the samples of rotaviruses circulating in pig herds of the São Paulo State present genotypes consistent with those described in the literature for Brazil and in other countries.

Conflict of Interests

The authors report no conflict of interests, and they have no direct financial relation with the commercial identities mentioned in the paper.

Acknowledgment

The authors would like to thank the FAPESP (São Paulo Research Foundation) for financial support (Process no. 2010/13652–8).

References

[1] M. K. Estes and A. Z. Kapikian, “Rotaviruses,” in Fields Virology, B. N. Fields, P. M. Knipe, and P. M. Howley, Eds., pp. 1917–1973, Lippincott Williams & Wilkins, Philadelphia, Pa, USA, 5th edition, 2007.
[2] A. A. Alfieri, A. F. Alfieri, and E. A. Beuttemmüller, “Rotavirose suina: tópicos sobre etiologia, infecção e controle,” Semina: Ciências Agrárias, vol. 20, no. 1, pp. 90–97, 1999.
[3] Paho: Pan American Health Organization, “Rotaviral gastroenteritis,” in Zoonoses and Communicable Diseases Common to Man and Animals: Chlamydiases, Rickettsioses and Viruses, pp. 286–294, PAHO, Washington, DC, USA, 3rd edition, 2001.
[4] L. J. Saif and B. Jiang, “Nongroup A rotaviruses of humans and animals,” Current Topics in Microbiology and Immunology, vol. 185, pp. 339–371, 1994.
[5] V. Martella, K. Bányai, J. Matthijnssens, C. Buonavoglia, and M. Ciarlet, “Zoonotic aspects of rotaviruses,” Veterinary Microbiology, vol. 140, no. 3–4, pp. 246–255, 2010.
[6] A. M. Q. King, M. J. Adams, E. B. Carstens, and E. J. Lefkowitz, Eds., Virus Taxonomy: Classification and Nomenclature of Viruses, Elsevier Academic Press, San Diego, Calif, USA, 9th edition, 2012.
[7] P. Schuck, Z. Taraporewala, P. McPhie, and J. T. Patton, “Rotavirus nonstructural protein NSP2 self-assembles into octamers that undergo ligand-induced conformational changes,” The Journal of Biological Chemistry, vol. 276, no. 13, pp. 9679–9687, 2001.
[8] Z. F. Taraporewala and J. T. Patton, “Identification and characterization of the helix-destabilizing activity of rotavirus nonstructural protein NSP2,” Journal of Virology, vol. 75, no. 10, pp. 4519–4527, 2001.
[9] T. Bar-Magen, E. Spencer, and J. T. Patton, “An ATPase activity associated with the rotavirus phosphoprotein NSP5,” Virology, vol. 369, no. 2, pp. 389–399, 2007.
[10] L. Hu, D. C. Chow, J. T. Patton, T. Palzkill, M. K. Estes, and B. V. V. Prasad, “Crystallographic analysis of rotavirus NSP2-RNA complex reveals specific recognition of 5′ GG sequence for RTPase activity,” Journal of Virology, vol. 86, no. 19, pp. 10547–10557, 2012.
[11] I. Afrikanova, E. Fabbretti, M. C. Miozzo, and O. R. Burrone, “Rotavirus NSP5 phosphorylation is up-regulated by interaction with NSP2,” Journal of General Virology, vol. 79, no. 11, pp. 2679–2686, 1998.
[12] M. Berois, C. Sapin, I. Erk, D. Poncet, and J. Cohen, “Rotavirus nonstructural protein NSP5 interacts with major core protein VP2,” Journal of Virology, vol. 77, no. 3, pp. 1757–1763, 2003.
[13] C. Eichwald, G. Jacob, B. Muszynski, J. E. Allende, and O. R. Burrone, “Uncoupling substrate and activation functions of rotavirus NSP5: phosphorylation of Ser-67 by casein kinase I is essential for hyperphosphorylation,” Proceedings of the National Academy of Sciences of the United States of America, vol. 101, no. 46, pp. 16304–16309, 2004.
[14] C. Eichwald, J. F. Rodríguez, and O. R. Burrone, “Characterization of rotavirus NSP2/NSP5 interactions and the dynamics of viroplasm formation,” Journal of General Virology, vol. 85, no. 3, pp. 625–634, 2004.
[15] J. J. Carreño-Torres, M. Gutiérrez, C. F. Arias, S. López, and P. Isa, “Characterization of viroplasm formation during the early stages of rotavirus infection,” Virology Journal, vol. 7, article 350, 2010.
[16] A. Sen, D. Agresti, and E. R. Mackow, “Hyperphosphorylation of the rotavirus NSP5 protein is independent of serine 67, NSP2, or the intrinsic insolubility of NSP5 and is regulated by cellular phosphatases,” Journal of Virology, vol. 80, no. 4, pp. 1807–1816, 2006.
[17] M. A. Torres-Vega, R. A. González, M. Duarte, D. Poncet, S. López, and C. F. Arias, “The C-terminal domain of rotavirus NSP5 is essential for its multimerization, hyperphosphorylation and interaction with NSP6,” Journal of General Virology, vol. 81, no. 3, pp. 821–830, 2000.
[18] M. Samaniego-Hernández, A. León-Rodríguez, R. Aparicio-Fabre, C. Arias-Ortiz, and A. P. Barba de La Rosa, “Expression and purification of rotavirus proteins NSP5 and NSP6 in Escherichia coli,” Cell Biochemistry and Biophysics, vol. 44, no. 3, pp. 336–341, 2006.
[19] E. W. Rainsford and M. A. McCrae, “Characterization of the NSP6 protein product of rotavirus gene II,” Virus Research, vol. 130, no. 1-2, pp. 193–201, 2007.
[20] J. Matthijnssens, M. Ciarlet, S. M. Mcdonald et al., “Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG),” Archives of Virology, vol. 156, no. 8, pp. 1397–1413, 2011.
[21] J. Matthijnssens, M. Rahman, V. Martella et al., “Full genomic analysis of human rotavirus strain B4106 and lapine rotavirus
strain 30/96 provides evidence for interspecies transmission,” *Journal of Virology*, vol. 80, no. 8, pp. 3801–3810, 2006.

[22] T. Tsugawa and Y. Hoshino, “Whole genome sequence and phylogenetic analyses reveal human rotavirus G3P[3] strains Rol845 and HCR3A are examples of direct virion transmission of canine/feline rotaviruses to humans,” *Virology*, vol. 380, no. 2, pp. 344–353, 2008.

[23] P. Khamrin, N. Maneekarn, R. Malasao et al., “Genotypic linkages of VP4, VP6, VP7, NSP4, NSP5 genes of rotaviruses circulating among children with acute gastroenteritis in Thailand,” *Infection, Genetics and Evolution*, vol. 10, no. 4, pp. 467–472, 2010.

[24] N. Cook, J. Bridger, K. Kendall, M. I. Gomara, L. El-Attar, and J. Gray, “The zoonotic potential of rotavirus,” *Journal of Infection*, vol. 48, no. 4, pp. 289–302, 2004.

[25] A. Steyer, M. Poljšak-Prijatelj, D. Barlič-Maganja, and J. Marin, “Human, porcine and bovine rotaviruses in Slovenia: evidence of interspecies transmission and genome reassortment,” *Journal of General Virology*, vol. 89, no. 7, pp. 1690–1698, 2008.

[26] V. Martella, M. Ciarlet, R. Baselga et al., “Sequence analysis of the VP7 and VP4 genes identifies a novel VP7 gene allele of porcine rotaviruses, sharing a common evolutionary origin with human G2 rotaviruses,” *Virology*, vol. 337, no. 1, pp. 111–123, 2005.

[27] A. Mukherjee, D. Dutta, S. Ghosh et al., “Full genomic analysis of a human group A rotavirus G9P[6] strain from Eastern India provides evidence for porcine-to-human interspecies transmission,” *Archives of Virology*, vol. 154, no. 5, pp. 733–746, 2009.

[28] A. N. B. Salem, A. C. Sergei, P. B. Olga, G. A. Olga, A. Mahjoub, and B. P. Larissa, “Multiplex nested RT-PCR for the detection of porcine enteric viruses,” *Journal of Virological Methods*, vol. 165, no. 2, pp. 283–293, 2010.

[29] T. A. Hall, “BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT,” *Nucleic Acids Symposium Series*, vol. 41, pp. 95–98, 1999.

[30] M. A. Larkin, G. Blackshields, N. P. Brown et al., “Clustal W and Clustal X version 2.0,” *Bioinformatics*, vol. 23, no. 21, pp. 2947–2948, 2007.

[31] S. F. Altschul, W. Gish, W. Miller, E. W. Myers, and D. J. Lipman, “Basic local alignment search tool,” *Journal of Molecular Biology*, vol. 215, no. 3, pp. 403–410, 1990.

[32] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar, “MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods,” *Molecular Biology and Evolution*, vol. 28, no. 10, pp. 2731–2739, 2011.

[33] J. T. Patton, L. Salter-Cid, A. Kalbach, E. A. Mansell, and M. Kattoura, “Nucleotide and amino acid sequence analysis of the rotavirus nonstructural RNA-binding protein NS35,” *Virology*, vol. 192, no. 2, pp. 438–446, 1993.

[34] M. Kumar, H. Jayaram, R. Vasquez-Del Carpio et al., “Crystallographic and biochemical analysis of rotavirus NSP2 with nucleotides reveals a nucleoside diphosphate kinase-like activity,” *Journal of Virology*, vol. 81, no. 22, pp. 12272–12284, 2007.

[35] P. H. Sotelo, M. Schümann, E. Krause, and J. Chnaiderman, “Analysis of rotavirus non-structural protein NSP5 by mass spectrometry reveals a complex phosphorylation pattern,” *Virus Research*, vol. 149, no. 1, pp. 104–108, 2010.

[36] F. Gregori, C. A. R. Rosales, P. E. Brandão, R. M. Soares, and J. A. Jerez, “Diversidade genotípica de rotavírus suínos no Estado de São Paulo,” *Pesquisa Veterinária Brasileira*, vol. 29, pp. 707–712, 2009.