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Towards Comprehensive Molecular Investigation of *Rotavirus A* Prevalence in Neonatal Calves and Pregnant Cows

[1] Pham Hong Son
[1] Hue University, Member University of Agriculture and Forestry, Vietnam
[1] Phung Hung, Hue city, Thua Thien Hue province, Vietnam

Email: phongson@hueuni.edu.vn; Corresponding Author: **Pham Hong Son**

Abstract
Searching for the presence of species *Rotavirus A*, or Group A rotaviruses (RVA), in clinical samples could be essential for confirming diagnosis on the etiologic agent, and Reverse Transcription PCR (RT-PCR) amplification of the virus' capsid VP6 gene in animal feces is the first choice as the protein is considered as the determinant of species. For that purpose, we have applied several adopted primer pairs with fecal samples from local calves and cows and obtained mostly negative outcomes. The formation non-specific "trash" bands of PCR products has prompted us to check capacity of the pairs in amplifying the species-specific gene. By screening the sameness/conservativeness of VP6 gene sequences of RVA bovine/cow strains available in GenBank Database we have chosen several fragments that can serve as consensus primers for detection of almost all of the already sequenced strains of the species. The theoretical outcomes were much increased, meanwhile the RT-PCR products of fecal samples proved that the pair of primers worked well in amplification of the target fragment of the gene. Thus, the capacity of VP6 gene-amplifying RT-PCR could be broadened for more comprehensive epidemiological investigation of the viral infections.

Keywords: Consensus Primer, Conservativeness, Diarrhea, Rotavirus, RT-PCR

1. Introduction

The species *Rotavirus A* (RVA), specified also as "group A rotaviruses" or "type A rotaviruses", belongs to the genus *Rotavirus* (RV) of nine species, namely, A, B, C, D, F, G, H, I, and J groups/types on the basis of the genetic property of VP6 [1]. Regarding to structure, the genus is a group of non-enveloped viruses [2], having virions with three layers of six original structural proteins, namely VP1 to VP4, VP6 and VP7. Amongst them the surficial spike protein VP4 of the outer capsid layer is often enzymatically cleaved into VP5 (designated also as VP5*) and VP8 (also as VP8*) initiating the virion's attachment to host cell. Besides, in host cells infected with the virus there are six non-structural proteins, designated as NSP1 to NSP6 [3, 4]. Inside each virion usually up to 76.5 nm in diameter [5, 6], there is a double-stranded RNA genome consisting of 11 fragments acting as 11 genes being in close contact with proteins VP1, VP2 and VP3 [7] and responsible for encoding the 12 original proteins. Amongst the gene fragments, the smallest one, namely fragment 11, transcribes in two different ways to translate into two non-structural proteins NSP5 and NSP6 [8]. Thus, for detection of the viral species we can choose to demonstrate the presence of either one of the 12 proteins, the presence of their relevant genes or antibodies specific to them in clinical samples as well as any combination of them. Of the proteins, the virion intermediate protein, or the capsid inner layer protein, designated as VP6, serves as the determinant for virus species classification [1] meanwhile the two surficial proteins VP4 and VP7 serve the determinants of respectively P (protease-cleavable) type [9] and G (glycoprotein) type [10] of the species. So, detection of the genes or antigens of VP6 and relevant products originated from it could be the first choices for confirming the presence of RVA, then reference to surficial proteins VP4 and VP7 of the virions. These procedures are useful for diagnosis of the viral diarrhea in neonatal and young humans and animals, important for the sake of their health as the viruses have long been considered as the etiologic agents causing more than 90% rotavirus infections in humans [11] leading usually to diarrhea in infants and young children [12] and as a major cause of diarrhea in wild and reared animals with high morbidity and mortality rates in young calves and piglets [13]. This species was investigated in many countries as the most common cause affecting almost one third of children hospitalized with severe diarrhea [14]. In calves reared in America, prevalence rate of the infection reported in 1979 was 98.1% (SNT – serum neutralization test) [15] and in 1995 was 44% (RT-PCR) [16], in the Netherlands in

1980 was 46% (ELISA – enzyme-linked immunosorbent assay) [17], in Australia in 1985 was 49% (PAGE – polyacrylamide gel electrophoresis) [18] and in 1992 was 48.7% (ELISA) [19], in Italy in 1988 was 90% (SNT – serum neutralization test) [20] and in 2005 was 16.8% (ELISA) [21], in Sri-Lanka in 1995 was 68.5% (ELISA) [22], in Canada in 1995 was 26.4% (ELISA) [23], in Japan in 1998 was 16.7% (RT-PCR) [24], in Argentina in 2006 was 62.5% (RT-PCR) [25], in Ireland in 2006 was 91% (ELISA) [26]… And, even "Rotavirus is commonly isolated from diarrheic calves and pigs" [27]. The viruses are also considered as zoonotic as RVA strains from animals can either transmit directly to humans [28] or supply one or more RNA segments for genome reassortment in human strains forming more infective strains [29, 31], investigation of virus prevalence in animals could be useful for prevention of the infections in humans. However, because of the diversity of the species, such as the nucleotide percentage identity cut-off values based on genome fragment VP6 could define rotavirus into 11 I (inner capsid protein) genotypic groups with the percentage identity cut-off values of 85% [32], so, with 85% identity, or otherwise 15% discrepancies, there should be many different sequences amongst RVA species. So, RT-PCR detection based on VP6 gene template could with high probability result in "failure" while using strain-specific oligonucleotide primer pairs presented by primer software automatically. The more specific primers are the less comprehensive the investigation could be. Hence, we have to design primers on the basis of conservativeness screening of nucleotide sequences for comprehensive investigation of the viral species. This paper presents some outcomes of experiments aiming to such consensus primer establishment.

2. Materials and methods

2.1 Adopting complete VP6 gene primers

From reference sources available we chose the pair of 61F (GGC TTT TAA ACG AAG TCT TC) and 62R (GGT CAC ATC CTC TCA CTA CG) previously [16] designed for complementary DNA (cDNA) synthesis of RVA VP6 complete gene to make template for nested PCR, since our check for their ability to amplify genes available in the GenBank database (NCBI) using the Primer Blast software [33] presented no gene of any other species except for a series of complete sequences of VP6 gene of RVA strains, which we could find by copying the results of Primer Blast searches, pasting to Microsoft (MS) Word files and checking key words with MS Word Navigation or "Finding".

2.2 Designing new VP6 gene primer pairs

Primer 63F (TTC AGG TCG CTG GAT TCG AC) and 64R (TTG CGT CGG CAA GTA CAG AT) were chosen from candidates designed on the basis of complete VP6 gene sequence of bovine RVA strain RVA/Yak-tc/CHN/HY-1/2018/G6P [11] (GenBank: MK250428.1) with the aid of the Primer Blast [33] (Fig. 1).

Meanwhile, the sequences of oligonucleotide primer pair of 65F and 66R were determined by screening the most conservative primer candidate fragments using Nucleotide BLAST: Align two or more sequences using blast of NCBI, USA (Fig. 2).

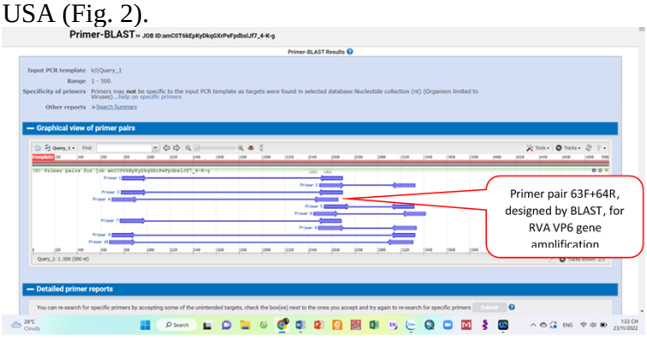

Fig 1: A primer pair number 4 was chosen amongst 10 suggested by Primer Blast, and designed as 63F+64R.

Amongst several conservative candidates, as a result, we have chosen pair of 65F (GAT GCT AGA GAC AAA ATT GTC GAA GG) and 66R (CAG CTG AAT TAA TCA CTC TTG GAA AAC) to be tested. The main properties they have are listed in Table 1.

Table 1: Major properties of utilized primers for VP6 gene amplification

Primers	Length	Tm	GC%	Self-complementarity	3' Self-complementarity
61F	20	52.84	40.00	7.00	7.00
62R	20	57.15	55.00	3.00	2.00
63F	20	60.04	50.00	5.00	**2.00**
64R	20	60.11	55.00	4.00	**2.00**
65F	26	60.46	42.31	8.00	**2.00**
66R	27	59.46	37.04	6.00	**0.00**

2.3 Sampling

Fecal samples from calves and gestating cows of both healthy and diarrheic appearance were collected, each in a plastic bag, kept cool on ice while transported to laboratory to be tested, or kept at -10 ºC to -20 ºC in delayed cases of tests. The minimum numbers of samples in each group were calculated for statistical validity as n = z^2×(p(1-p))/d^2, in which 95% confidence limit coefficient z = 1.96, estimated prevalence p = 0.9, and permitted error d = 0.1, which was from 35 in each group. The real numbers of samples did depend on the epidemiological situation of diarrhea in bovine.

2.4 RT-PCR amplification of RVA genes

Virus RNA extraction was done with commercially available kits. RNA/DNA TopPure Fluid Viral Extraction Kits (ABT Corp.) were used according to the provider's instructions to obtain 50 µL of eluates containing solution of DNA and RNA. For that, about 3 - 4 g of each fecal samples were emulsified in 1 mL distilled water, then 1 mL of the emulsion was transferred into an Eppendorf tube and centrifuged at 6000 x*g* for 5 min, and 200 µL of the supernatants were subjected to the TopPure extraction procedures. Concurrently, 200 µL of 1:4 diluted RotaTeq vaccine (Merck Sharp & Dohme Corp.) were also subjected to the same procedures for obtaining TopPure-extracted nucleotide solutions.

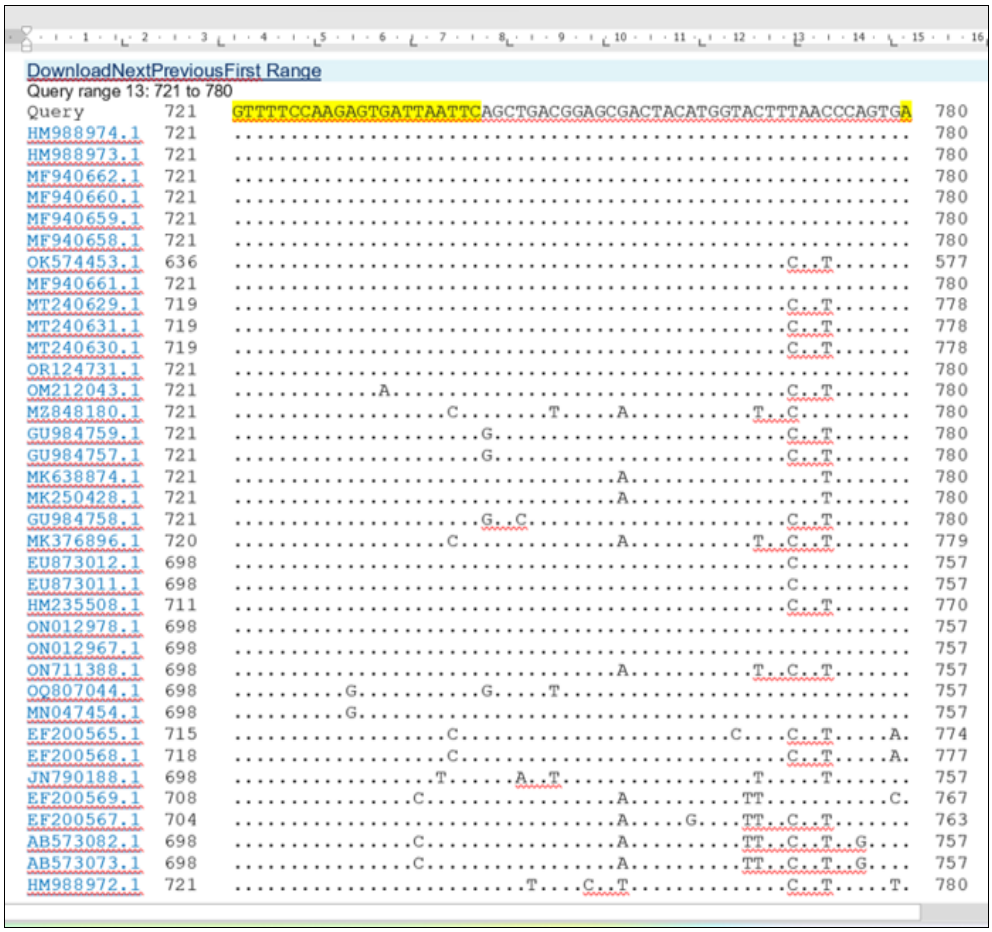

Fig 2: A representative print-screen picture showing part of conservative sequences of target VP6 gene that could serve as a candidate for constructing primer pairs of 65F+66R to be used in RVA detection PCR. (Sequences longer than 20 nucleotide covering identical nucleotides for most GenBank-available bovine RVA sequences were chosen and paired for product sizes in the range of optimal amplification of about 400 bps to 700 bps, of which the second primer candidates were then conversed complementarily and checked for melting points with the aid of Blast Primer of NCBI, USA.)

RT-PCR amplification of virion structural protein specific gene fragments were carried out in two steps. The first was of reverse transcription (RT) reaction which catalyzes cDNA synthesis and then PCR amplification of the obtained cDNA with *Taq* polymerase with several primer pairs. Of them, primer 63F and 64R were designed on the basis of complete VP6 gene sequence of a bovine RVA strain as mentioned and chosen because of reasonable size of PCR products and with no gene termini involved (Fig.1). The sequences of oligonucleotide primer pair of 65F and 66R, otherwise, were determined by screening possible conservative fragments, i.e., those fragments longer than 20 bases with the same nucleotide sequences present in almost all of the bovine/cow strains available in GenBank database (Fig. 2), as proper candidates for primers. Of each chosen couple of fragments, the second one was conversed complementarily with Mega-X [34] and checking with the aid of the Primer Blast of USA NIH (https://www.ncbi.nlm.nih.gov/guide/howto/design-pcr-primers/) for their RVA specificity. This pair of primers, i.e., 65F+66R, was chosen to test and presented here because they have reasonable values of melting temperatures (both are around 60 ºC). Used in this research were also other pair of primers, which were adopted from previous researchers' published articles, namely 61F+62R for VP6 complete gene of 1356 bp length [16].

The TopPure-extracted fecal sample nucleotide solutions as well as those of RotaTeq vaccine were treated at 95 ºC for 5 min for denaturation of double-stranded RNA followed by chilling on ice at least for 5 min, and used as templates for synthesizing cDNA with a GoScript Reverse Transcriptase kit according to the instructions of the provider (Promega Corp.). For that, into each 0.2-mL tube, 0.5 µL forward primer (10 pmol), 0.5 µL reverse primer (10 pmol), 1 µL DEPC-treated water, and 1 µL of the denatured nucleic acids of a sample, as template solution, were added. After being incubated at 95 ºC for 5 min and immediately placed on ice for at least 5 min, these tubes of reaction were centrifuged briefly and placed back onto ice water, then, for creation of cDNA, to each of the tubes were added 9 µL of transcription mixture, which was prepared as follows. For the cases of up to 83 reactions, preparation of a batch of 750 µL of the transcription mixture was implemented. For that, we mixed 200 µL GoScript 5× reaction buffer, 50 µL PCR nucleotide mix of four deoxyribonucleoside triphosphates (dNTPs) with 10 mM concentration each, 100 µL 25 mM $MgCl_2$, 50 µL GoScript Reverse Transcriptase (160 units/µL) with and 350 µL DEPC-treated water. After adding 9 µL of this transcription mixture into each of the reaction tubes, the latter (containing 12 µL each) were incubated at 25 ºC for 5 min then at 42 ºC for 1 h. The product mixtures were ready as cDNA template solutions. For PCR amplification, EZ PCR mix (Phusa Genomics Corp., https://www.phusagenomics.com/) containing *Taq* polymerase and dNTPs (Thermo Fisher Scientific) in a 0.2-mL tube was used. To each of these tubes, 25 µL 2× PCR

buffer, 1 µL forward primer (20 pmol), 1 µL reverse primer (20 pmol), and 13 µL DEPC-treated water were added. After being mixed and then briefly centrifuged, 8 µL each from this mixture were pipetted into another 0.2-mL tube, into which 2 µL of each of the above-prepared cDNA template solutions of either samples or RotaTeq vaccine were then added. The tube was briefly centrifuged to settle the contents for PCR amplification, which was performed on an Axygen MaxyGene II Thermal Cycler (Corning), beginning with one cycle of reverse transcription at 45 ºC for 20 min and one cycle of polymerase activation at 95 ºC for 20 min, then 40 cycles of combination of three levels of temperatures, including 95 ºC for 30 s, 62 ºC for 30 s, and 72 ºC for 1 min. An additional elongation step of 5 min at 72 ºC was applied.

The content of each PCR tube was mixed with 2 µL 6× GelRed loading buffer tricolor (ABT/Biotium Inc.,) and electrophoresed through 1% Agarose M (Bio Basic) gel in 1× TAE buffer solution (Bio Basic). Or otherwise, into the ten µL of RT-PCR tube were added 2 µL 6× loading buffer, mixed, and electrophoresed through agarose gel already stained with Safe Dye (P-Sdye-0250, Phusa Genomics, 5 µL mixed with 100 mL of agarose solution prior to casting the gel). Meanwhile, in some gels, RotaTeq vaccine virus' RT-PCR products were also run alongside with samples, serving as the internal control (IC) reference. The electrophoresed gels were screened for RT-PCR products and DNA marker bands under a UV source and recorded with cameras behind a UV shield.

2.4 Data analysis

For checking the number of products amplifiable with one or another pair of primers, the primer sequences were search on Primer Blast and run for the possible products. The contents of each Blast file were copied into a Microsoft Word file and check for the appearance of target key words (bovine/cow) using "Finding" function of the software.

3. Results

3.1 Possibility of creation of comprehensive primers for RVA VP6 gene fragment amplification

The capacity of primer pairs 65F and 66R in annealing to RVA strains' VP6 gene sequences were demonstrated in Table 2 and Table 3. Primer 65F has percentage identity of 100% with 29 amongst 49 databased strains (59.2%) in sense of linear alignment. The rest strains have a range of identity percentage from 88.46% to 96.15%.

Table 2: Capacity of primer 65F in amplification of RVA VP6 gene according to Blastn alignment

	Accession	Scientific Name	Max Score	Total Score	Query Cover	E value	Percentage of Identity	Accession Lengths
1.	OR124731.1	Bovine rotavirus	52.0	181	100%	2,00E-10	**100.00%**	1356
2.	MZ848180.1	Rotavirus A	52.0	195	100%	2,00E-10	**100.00%**	1356
3.	MK638874.1	Bovine rotavirus A	52.0	240	100%	2,00E-10	**100.00%**	1356
4.	MK250428.1	Bovine rotavirus A	52.0	240	100%	2,00E-10	**100.00%**	1356
5.	MF940662.1	Bovine rotavirus	52.0	210	100%	2,00E-10	**100.00%**	1356
6.	MF940661.1	Bovine rotavirus	52.0	210	100%	2,00E-10	**100.00%**	1356
7.	MF940660.1	Bovine rotavirus	52.0	210	100%	2,00E-10	**100.00%**	1356
8.	MF940659.1	Bovine rotavirus	52.0	210	100%	2,00E-10	**100.00%**	1356
9.	MF940658.1	Bovine rotavirus	52.0	210	100%	2,00E-10	**100.00%**	1356
10.	**HM988974.1**	Bovine rotavirus A	52.0	210	100%	2,00E-10	**100.00%**	1356
11.	HM988973.1	Bovine rotavirus A	52.0	224	100%	2,00E-10	**100.00%**	1356
12.	GU984759.1	Bovine rotavirus A	52.0	212	100%	2,00E-10	**100.00%**	1356
13.	GU984758.1	Bovine rotavirus A	52.0	197	100%	2,00E-10	**100.00%**	1356
14.	GU984757.1	Bovine rotavirus A	52.0	212	100%	2,00E-10	**100.00%**	1356
15.	OK574453.1	Bovine rotavirus	52.0	238	100%	2,00E-10	**100.00%**	1356
16.	MK376896.1	Rotavirus A	52.0	152	100%	2,00E-10	**100.00%**	1355
17.	MT240631.1	Bovine rotavirus A	52.0	238	100%	2,00E-10	**100.00%**	1354
18.	MT240630.1	Bovine rotavirus A	52.0	238	100%	2,00E-10	**100.00%**	1354
19.	MT240629.1	Bovine rotavirus A	52.0	238	100%	2,00E-10	**100.00%**	1354
20.	HM235508.1	Bovine rotavirus	52.0	197	100%	2,00E-10	**100.00%**	1339
21.	ON711388.1	Bovine rotavirus A	52.0	212	100%	2,00E-10	**100.00%**	1302
22.	EF200565.1	Bovine rotavirus A	52.0	230	100%	2,00E-10	**100.00%**	1299
23.	OQ807044.1	Bovine rotavirus	52.0	166	100%	2,00E-10	**100.00%**	1194
24.	ON012978.1	Bovine rotavirus A	52.0	195	100%	2,00E-10	**100.00%**	1194
25.	ON012967.1	Bovine rotavirus A	52.0	195	100%	2,00E-10	**100.00%**	1194
26.	MN047454.1	Bovine rotavirus A	52.0	206	100%	2,00E-10	**100.00%**	1194
27.	EU873012.1	Bovine rotavirus	52.0	210	100%	2,00E-10	**100.00%**	1194
28.	EU873011.1	Bovine rotavirus	52.0	210	100%	2,00E-10	**100.00%**	1194
29.	AB738416.1	Bovine rotavirus C	14.4	57.5	**61%**	**48**	**100.00%**	1352
30.	OM212043.1	Bovine rotavirus A	44.1	216	100%	5,00E-08	96.15%	1356
31.	EF200569.1	Bovine rotavirus A	44.1	130	100%	5,00E-08	96.15%	1292
32.	EF200568.1	Bovine rotavirus A	44.1	222	100%	5,00E-08	96.15%	1292
33.	AB573082.1	Bovine rotavirus A	44.1	161	100%	5,00E-08	96.15%	1194
34.	AB573073.1	Bovine rotavirus A	44.1	132	100%	5,00E-08	96.15%	1194
35.	EF200567.1	Bovine rotavirus A	32.2	118	**76%**	2,00E-04	95.00%	1288
36.	MF940717.1	Bovine rotavirus	36.2	140	100%	1,00E-05	92.31%	1356
37.	MF940716.1	Bovine rotavirus	36.2	126	100%	1,00E-05	92.31%	1356
38.	MF940715.1	Bovine rotavirus	36.2	126	100%	1,00E-05	92.31%	1356
39.	MF940714.1	Bovine rotavirus	36.2	140	100%	1,00E-05	92.31%	1356
40.	MF940713.1	Bovine rotavirus	36.2	140	100%	1,00E-05	92.31%	1356

	Accession	Scientific Name	Max Score	Total Score	Query Cover	E value	Percentage of Identity	Accession Lengths
41.	MF940607.1	Bovine rotavirus	36.2	126	100%	1,00E-05	92.31%	1356
42.	MF940606.1	Bovine rotavirus	36.2	126	100%	1,00E-05	92.31%	1356
43.	MF940605.1	Bovine rotavirus	36.2	126	100%	1,00E-05	92.31%	1356
44.	MF940604.1	Bovine rotavirus	36.2	126	100%	1,00E-05	92.31%	1356
45.	MF940603.1	Bovine rotavirus	36.2	126	100%	1,00E-05	92.31%	1356
46.	KF500178.1	Bovine rotavirus	36.2	140	100%	1,00E-05	92.31%	1356
47.	JN790188.1	Bovine rotavirus	36.2	257	100%	1,00E-05	92.31%	1194
48.	EU873010.1	Bovine rotavirus	36.2	124	100%	1,00E-05	92.31%	1194
49.	HM988972.1	Bovine rotavirus A	28.2	132	100%	0.003	88.46%	1356

Meanwhile, primer 66F has percentage identity of 100% with 24 amongst 49 databased strains (49.0%). The rest strains have a range of identity percentage from 92.59% to 96.3%. All these percentage values are much higher than cut-off value of 85% which have been suggested for discrimination of rotavirus species [32].

Table 3: Capacity of primer 66F in amplification of RVA VP6 gene according to Blastn alignment

	Accession	Scientific Name	Max Score	Total Score	Query Cover	E value	Percentage of Identity	Accession Lengths
1.	OR124731.1	Bovine rotavirus	54.0	205	100%	6,00E-11	100.00%	1356
2.	MK638874.1	Bovine rotavirus A	54.0	259	100%	6,00E-11	100.00%	1356
3.	MK250428.1	Bovine rotavirus A	54.0	259	100%	6,00E-11	100.00%	1356
4.	MF940662.1	Bovine rotavirus	54.0	216	100%	6,00E-11	100.00%	1356
5.	MF940661.1	Bovine rotavirus	54.0	216	100%	6,00E-11	100.00%	1356
6.	MF940660.1	Bovine rotavirus	54.0	216	100%	6,00E-11	100.00%	1356
7.	MF940659.1	Bovine rotavirus	54.0	216	100%	6,00E-11	100.00%	1356
8.	MF940658.1	Bovine rotavirus	54.0	216	100%	6,00E-11	100.00%	1356
9.	HM988973.1	Bovine rotavirus A	54.0	216	100%	6,00E-11	100.00%	1356
10.	**HM988974.1**	Bovine rotavirus A	54.0	216	100%	6,00E-11	100.00%	1356
11.	OK574453.1	Bovine rotavirus	54.0	203	100%	6,00E-11	100.00%	1356
12.	MT240631.1	Bovine rotavirus A	54.0	203	100%	6,00E-11	100.00%	1354
13.	MT240630.1	Bovine rotavirus A	54.0	203	100%	6,00E-11	100.00%	1354
14.	MT240629.1	Bovine rotavirus A	54.0	203	100%	6,00E-11	100.00%	1354
15.	HM235508.1	Bovine rotavirus	54.0	187	100%	6,00E-11	100.00%	1339
16.	ON711388.1	Bovine rotavirus A	54.0	212	100%	6,00E-11	100.00%	1302
17.	EF200567.1	Bovine rotavirus A	54.0	185	100%	6,00E-11	100.00%	1288
18.	ON012978.1	Bovine rotavirus A	54.0	216	100%	6,00E-11	100.00%	1194
19.	ON012967.1	Bovine rotavirus A	54.0	216	100%	6,00E-11	100.00%	1194
20.	EU873012.1	Bovine rotavirus	54.0	197	100%	6,00E-11	100.00%	1194
21.	EU873011.1	Bovine rotavirus	54.0	197	100%	6,00E-11	100.00%	1194
22.	HM988972.1	Bovine rotavirus A	52.0	225	96%	2,00E-10	100.00%	1356
23.	GU984758.1	Bovine rotavirus A	44.1	179	92%	6,00E-08	100.00%	1356
24.	AB738416.1	Bovine rotavirus C	18.3	75.8	**70%**	**3.3**	100.00%	1352
25.	OM212043.1	Bovine rotavirus A	46.1	226	100%	1,00E-08	96.30%	1356
26.	MZ848180.1	Rotavirus A	46.1	208	100%	1,00E-08	96.30%	1356
27.	MF940717.1	Bovine rotavirus	46.1	208	100%	1,00E-08	96.30%	1356
28.	MF940716.1	Bovine rotavirus	46.1	222	100%	1,00E-08	96.30%	1356
29.	MF940715.1	Bovine rotavirus	46.1	208	100%	1,00E-08	96.30%	1356
30.	MF940714.1	Bovine rotavirus	46.1	208	100%	1,00E-08	96.30%	1356
31.	MF940713.1	Bovine rotavirus	46.1	208	100%	1,00E-08	96.30%	1356
32.	MF940607.1	Bovine rotavirus	46.1	208	100%	1,00E-08	96.30%	1356
33.	MF940606.1	Bovine rotavirus	46.1	208	100%	1,00E-08	96.30%	1356
34.	MF940605.1	Bovine rotavirus	46.1	208	100%	1,00E-08	96.30%	1356
35.	MF940604.1	Bovine rotavirus	46.1	208	100%	1,00E-08	96.30%	1356
36.	MF940603.1	Bovine rotavirus	46.1	208	100%	1,00E-08	96.30%	1356
37.	KF500178.1	Bovine rotavirus	46.1	208	100%	1,00E-08	96.30%	1356
38.	GU984759.1	Bovine rotavirus A	46.1	181	100%	1,00E-08	96.30%	1356
39.	GU984757.1	Bovine rotavirus A	46.1	181	100%	1,00E-08	96.30%	1356
40.	MK376896.1	Rotavirus A	46.1	222	100%	1,00E-08	96.30%	1355
41.	EF200565.1	Bovine rotavirus A	46.1	175	100%	1,00E-08	96.30%	1299
42.	EF200569.1	Bovine rotavirus A	46.1	189	100%	1,00E-08	96.30%	1292
43.	EF200568.1	Bovine rotavirus A	46.1	189	100%	1,00E-08	96.30%	1292
44.	MN047454.1	Bovine rotavirus A	46.1	208	100%	1,00E-08	96.30%	1194
45.	EU873010.1	Bovine rotavirus	46.1	193	100%	1,00E-08	96.30%	1194
46.	AB573082.1	Bovine rotavirus A	46.1	189	100%	1,00E-08	96.30%	1194
47.	AB573073.1	Bovine rotavirus A	46.1	175	100%	1,00E-08	96.30%	1194
48.	JN790188.1	Bovine rotavirus	42.1	159	92%	2,00E-07	96.00%	1194
49.	OQ807044.1	Bovine rotavirus	38.2	200	100%	4,00E-06	92.59%	1194

3.2 RT-PCR amplification of RVA VP gene fragments

The outcomes of RT-PCR amplification of RVA in the fecal samples with primer pair of 61F+62R are presented exemplarily in Fig. 3. Neither calves nor cows seemingly have shed the virus in their feces even whether they have been diarrheic or not.

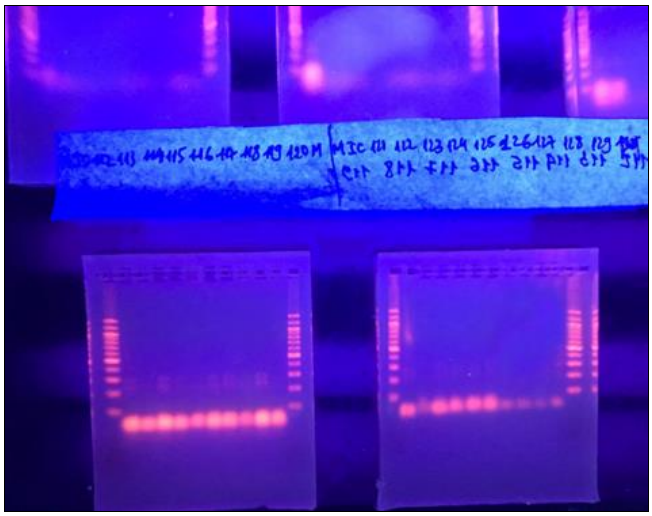

Fig 3: A representative picture showing all fecal samples from diarrheic and healthy calves and pregnant cows were negative. (PCR "trash products" shorter than those of target sequences may be the outcomes of high values of self-complementarity and 3'-self-complementarity in the absence of complementary templates.)

However, as shown in Fig. 4 and Fig 5., RT-PCR with primer pair 63F+64R as well as primer pair 65F+66R revealed that there was a diarrheic calf positive, that is, infected with RVA.

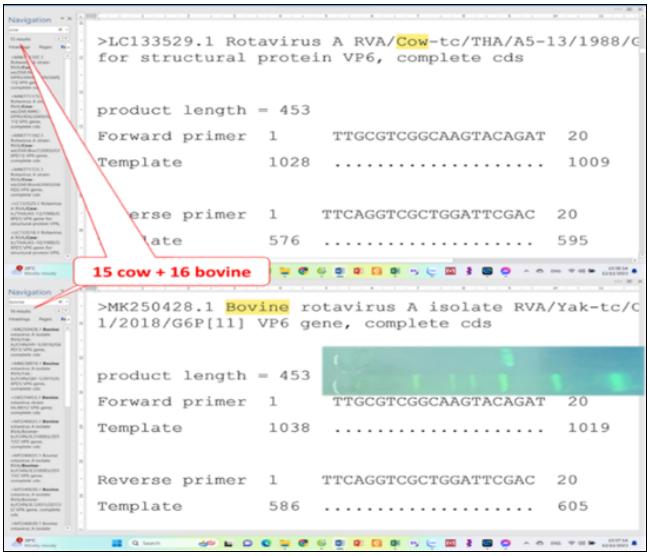

Fig 4: Capacity of primer pair 63F+64R. The keyword "bovine" appears 16 times, and that of "cow" 15 times, showing the number of strains that can be amplified with the pair of primers, which in comparison with the total number of 49 strains having databased nucleotide sequences counts for a small portion (32.7%) of detectable strains. The embedded picture shows the performance of the primer pair 63F+64R with a band of specific 453 bp length RT-PCR product from feces of the only diarrheic calf.

With the pairs of primers applied in RT-PCR for amplification of the tested fecal samples, we revealed that there was only one diarrheic calf infected with RVA but this infected animal could not be detected with the adopted primer pair for complete VP6 gene amplification (Fig. 3). The positive outcomes could be obtained with primer pair 63F+64R (Fig. 4), which was designed by Blast Primer, and with primer pair 65F+66R (Fig. 5), which was designed by drawing from screening conservative fragments of the target sequences. This showed that there was only one calf amongst 49 diarrheic calves positive or amongst 197 bovine individuals tested, i.e., 2.04% of diarrheic calves or 0.51% of cattle possessing the virus in their feces.

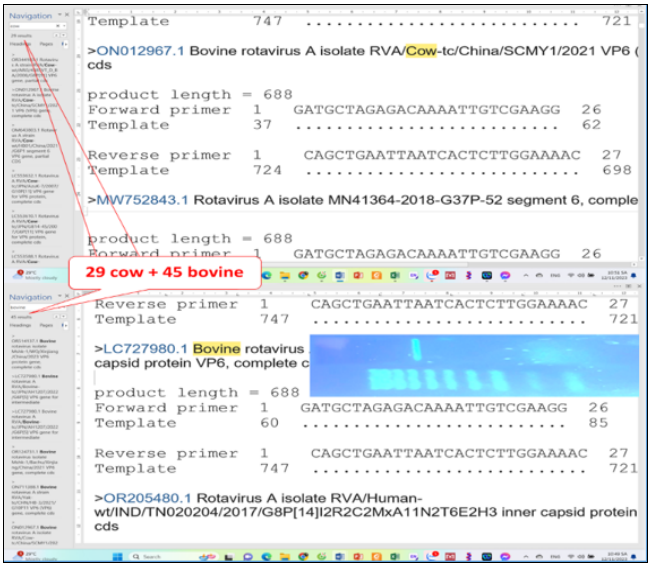

Fig 5: Capacity of primer pair 65F+66R The keyword "bovine" appears 45 times, and that of "cow" 29 times amongst total databased 49 rotavirus strains, shows improved ratios (respectively 91.8% and 59.2%) of strains that can be amplified with the pair of primers. The embedded picture shows the performance of the primer pair 65F+66R with a band of specific 688 bp length RT-PCR product from feces of the only diarrheic calf.

Related to the properties of pathogen, the double stranded ribonucleic acid (dsRNA) genome of RVA requires additional steps of denaturation of double stranded RNAs for reverse transcription (RT) of more fragile genome already denatured. Researches of these kinds could fail because of that. However, the positive outcomes we have obtained with primer pairs 63F+64R and 65F+66R in comparison with primer 61F+62R showed another phenomenon. Strain-specific primers cannot be used as consensus primers for epidemiological investigation. So, we should discriminate strain-specific primers from species-specific primers in algorithm of primer designing for different purposes of diagnosis confirmation, epidemiological comprehensive investigation and tracking one or another pathogenic strain. Furthermore, in cases of reasonable melting temperatures and low values of 3' self-complementarity, the values of primer self-complementarity can be as high as 8.

Technically, the different sensitivity of the RT-PCR tests applied with different primer pairs requires analysis of the properties of the primers. Of the double-layered capsid of rotavirus, proteins VP6 composing the capsid inner layer, and serving as the intermediate virion layer, are highly antigenic and have been used to identify rotavirus species [35] as well as to implement laboratory tests for determining RVA infections [36]. The negativity of the RVA detection outcomes might be because of their self-complementarity

and 3' self-complementarity in the absence of complementary templates, the large gaps of the melting temperatures (Tm) of primers each with other in each pair and each with the applied primer annealing temperatures (62 ^{o}C), and the lengthiness of the target sequences as well as utilization of terminal sequences for primers, as with the case of pair 61F+62R (1356 bp long target). The inherent mismatches in combination with strain-specific variation could make gene terminal primers designed on the base of databased sequences lead to failures. Terminal sequences of biological genera are often amplified with so-called genus consensus primers with some mismatching nucleotides, which we can check with inverse PCR in cases of DNA genome [37], and then serve as template for nucleotide sequencing. From the real performance and theory, the pair of primers 63F+64R, which were designed on the basis of VP6 gene complete sequence of a bovine RVA strain with the aid of the Primer Blast software [33], would be suitable for detecting RVA infection in cattle. However, for comprehensive epidemiological investigation we should design primers with more conservative sequences, and in case of the diversity of RVA we should combine several pairs of such primer pairs for consensus detection.

4. Conclusion

For comprehensive epidemiological investigation, conservative fragments of databased sequences could be serve as potential primer candidates. Since strain-specific primers cannot succeed in amplifying diverse analogous genes of the same pathogenic species, it should be considered to create algorithm for designing PCR primers. And, technically, PCR primers with the value of self-complementarity of 8 were tested applicable.

5. Disclosure of conflict of interest

There are no conflicts of interest to declare.

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