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Utilizing some structural protein-encoding genes, virion antigens, and hemagglutination property of *Rotavirus* for investigation of the viral infection in bovine

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ABSTRACT

The genus *Rotavirus* (RV) has been reported as zoonotic, highly prevalent in diarrheic neonates, and possibly activated by gestation. Searching RV prevalence in bovines and checking its presence during convalescence of the animals from the illness are useful for One-Health management. Tests detecting specific genes encoding the virus' capsid VP6 and VP4 with Reverse transcription PCR (RT-PCR) amplification as well as virus antigens with both Immuno-chromatographic assay (ICA) and Shifting assay of standardized direct hemagglutination inhibition (SSDHI) in fecal samples taken individually from both diarrheic and healthy calves and pregnant cows were implemented. The tests showed low percentages of diarrheic calves infected with the virus, implying insignificant contribution of the virus in bovine diarrhea. However, ICA tests which were then performed for checking the virus in feces of a genetically and antigenically proven infected diarrheic calf daily during the course of illness and daily through a week after diarrhea stopped showed that the virus had a role in causing the illness. Meanwhile, specific antibody titration with hemagglutination inhibition (HI) reactions implemented with serum samples of locally reared slaughtered and live cattle showed a significantly (P = 0.0008) higher rate of the virus infection. The outcomes of the tests showed that application of the system of HA-HI-SSDHI with the use of RotaTeq vaccine is feasible in investigation of RV though the targets are restricted to bovine-specific P[8] strains.

Introduction

The genus *Rotavirus* (RV) consists of nine species of the family *Reoviridae*, amongst which *Rotavirus* A (RVA) is the most common species causing more than 90% *Rotavirus* infections in humans (Leung *et al*., 2005). The virus is a major cause of diarrhea in wild and reared animals with high morbidity and mortality in young calves and piglets (Martella *et al*., 2010). So, for controlling the zoonosis, it could be useful to investigate the virus in bovines. Morphologically, the virus could be visualized under an electron microscope as round, wheel-like, or so-called rota-like, virions (Flewett *et al*., 1974) approaching 76.5 nm in diameter (Prasad and Chiu, 1994). Structurally, each virion consists of three layers of six original structural proteins, or viral proteins (VPs), namely VP1 to VP4, VP6 and VP7, and, in addition, there are six non-structural proteins, designated as NSP1 to NSP6, synthesized only in host cells infected with the virus (Jayaram *et al*., 2004). The core of each virion consists of molecules VP2 in close contact with VP1 and VP3 proteins and a double stranded RNA genome comprising 11 fragments acting as 11 genes (Trask *et al*., 2012). Amongst them, fragment 11 can be transcribed from two different initiation codons to translate into two non-structural proteins NSP5 and NSP6 (Kirkwood, 2010). Of the structural proteins, VP7 and VP4 are present on the virion surface, mutually interact each with another, and are targets of neutralizing antibodies (Clark *et al*., 1985). VP7 serves as G (glycoprotein) type determinants of the genus (O'Ryan *et al*., 1994), meanwhile VP4 determines the P (protease-cleavable) type (Hoshino *et al*., 2002) of the virus. To make virion infective, the surficial spike protein VP4 of the outer capsid layer is proteolysis-cleaved into VP5 and VP8 (Arias *et al*., 1996). *Rotavirus* capsid protein VP5* (designated also as VP5) "permeabilizes membranes" (Denisova *et al*., 1999). The VP8* (also as VP8) domain of VP4 mediates the binding of the virion to glycans of host cell surfaces (Dormitzer *et al*., 2002). Meanwhile, NSP4 acts as a viral endotoxin (Ball *et al*., 1996; Tafazoli *et al*., 2001), in combination with the activities of VP4 and VP7, leading to infection of the host animal accompanied with intestinal histopathologic changes, such as enterocyte vacuolization, villous blunting, villous atrophy, crypt hyperplasia, flattening of absorptive epithelium and reduced villus:crypt ratios (Carpio *et al*., 1981), and with functional changes in the tight junctions between enterocytes resulting in paracellular leakage in polarized epithelial cells (Tafazoli *et al*., 2001). As a virion's foundation layer supporting VP4 and VP7 in the outside, VP6 composes the virion intermediate layer or the capsid inner layer and serves as the determinant for virus species classification, and on the basis of the genetic property of VP6 the genus *Rotavirus* was classified into A, B, C, D, F, G, H, I, and J groups/types/species (Matthijnssens *et al*., 2012) with an attributed percentage cut-off value of nucleotide sequence identity for species discrimination equal to 85% (Matthijnssens *et al*., 2008). So, for confirmation of the viral infections, showing any proof on the presence of either one of the 12 proteins, any combination of them, the presence of their relevant genes, or antibodies specific to them in clinical samples is helpful, though of different significance. Spatially, molecules VP6 are encased entirely inside a layer of molecules VP4 and VP7 and responsible for the formation of species-specific shape and size properties, they thus have no direct interactions with host cell surfaces, and so, could evolve independently from the host responsive activities. It must be a most conservative component sustaining successful virion assembly, and is thus suitable for genus/species determination. On the opposite, VP4 and VP7 molecules, together forming outer layer of the virions, interact with host cell surfaces initiating cell infection, so they should evolve in accordance with the host cells' changes/variants, thus probably change faster than VP6. Detection or determination of the presence of VP6, so, should be most effective for RV infection investigation. However, analyses of nucleotide sequences of GenBank-available RV complete genomes showed that the gene encoding RVA VP6 in bovines alone is so very diverse, that many primer pairs of RT-PCR amplification of the gene should be applied simultaneously for comprehensive investigation (Pham, 2023a). For that

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reason, application as many as possible methods to detect a larger range of strains of the pathogen as possible is advisable. This study being implemented on cattle reared in Thua Thien Hue, a central province of Vietnam, from the end of 2021 to the middle of 2023, utilized several genetic, antigenic, and antibody-detecting tests to determine RV infection prevalence in diarrheic and healthy calves as well as in diarrheic and healthy pregnant cows, since gestation was suspected to change the sensitivity/tolerance of female animals to the virus, from which newly born calves could be vertically infected.

Materials and methods

Sampling

Fecal samples from calves and gestating cows of both diarrheic (excreting feces of watery to pasty consistency) and non-diarrheic illness were collected individually, each in a plastic bag, and kept cool on ice while transported to laboratory to be tested or kept at -10°C in delayed cases of analysis. The numbers of samples in the four groups of bovines were planned to be collected equally, and at least 35 in each group as this minimum number (n) of samples was calculated according to the formula of n = z^2*(p(1 - p))/d^2, in which z = 1.96 (95% confidence limit coefficient), estimated rate of prevalence p = 0.9 or 0.1, and permitted error d = 0.1. The real numbers of samples (planned to be higher than 35 targeting at lowering the permitted error value to 0.08 corresponding to a maximum of 55 samples in each group) and the duration of sampling did depend on the epidemiological situations of diarrhea in bovine: once a scour sample from a diarrheic calf was taken a fecal sample from a normal calf should be collected from the same locality. Simultaneously, the same numbers of diarrheic pregnant cows and normal pregnant cows in the area were identified as targets for fecal sampling so that the numbers of the four kinds of samples were not too different. A total of 197 fecal samples were collected, amongst them 49 were from diarrheic calves, 49 from normal, 50 from diarrheic pregnant cows, and 49 from normal pregnant cows.

Blood serum samples for obtaining sera to be tested were collected from locally reared adult cattle other than those from which fecal samples were collected, most of which were collected at slaughtering moments in several local (Phu Duong, Thuy Duong, Thuy Chau, Chan May and Huong Hoa) abattoirs located in Thua Thien Hue province, Viet Nam, with the aid of slaughterhouse veterinary sanitary inspectors in large volumes foreseen for possible HI positive cases to utilize as antibody sources for SSDHI tests. As the numbers of bovine serum samples collected in each of the two consecutive years were higher than the minimum values of sample quantities planned to be collected as theoretically calculated with the above-mentioned formula for feces sampling, they were intendedly divided into two stage groups for broadening estimation ranges in comparative analysis.

RT-PCR amplification of RVA genes

Virus RNA extraction was performed with commercially available kits as described recently (Pham, 2023a). RNA/DNA TopPure Fluid Viral Extraction Kits (ABT Corp.) were used according to the provider's instructions to obtain 50 µL of eluates of nucleic acid solutions from 200 µL of emulsion centrifugation supernatants of 3 - 4 g of each fecal samples in 1 mL distilled water. In addition, 200 µL of 1:4 diluted RotaTeq vaccine (Merck Sharp & Dohme Corp.) were also subjected to the same TopPure extraction procedures for obtaining 50 µL TopPure-extracted nucleic acid solutions for internal control (IC) RT-PCR amplification.

RT-PCR amplification of virus structural protein-specific gene fragments were carried out in two steps of reverse transcriptase (RT)-catalyzed cDNA synthesis and then Taq polymerase-catalyzed PCR amplification of the obtained cDNA. Several targets, including three VP6 gene fragments and two VP4 gene fragments of RV were amplified for detection of their analogues possibly present in the fecal samples with several primer pairs adopted from previous authors. Of them, Con2+Con3 targeting 877 bp and Con2+OSU targeting 502 bp long fragments (Gouvea *et al*., 1994) of the VP4 gene, meanwhile primer pair 61F+62R targeting a complete sequence of 1356 bp (Chinsangaram *et al*., 1995) and 63F+64R and 65F+66R targeting respective 453 bp and 688 bp long fragments (Pham, 2023a) of the VP6 gene, were used for relevant gene amplification, as shown in Table 1.

The above-mentioned TopPure-extracted nucleotide solutions from the fecal samples as well as from RotaTeq vaccine were heated for 5 min at 95°C 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 (Promega Corp.) according to the protocol supplied by the provider. For that, 1 µL DEPC-treated water, 0.5 µL forward primer (10 pmol/25 pmol), 0.5 µL reverse primer (10 pmol/25 pmol), and 1 µL of the denatured nucleic acids of a sample, as template solution, were mixed in a 0.2-mL tube for each sample test. After being incubated at 95°C 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, into each of these tubes 9 µL of transcription mixture, prepared as shown previously (Pham, 2023a) were added and incubated to be cDNA templates ready for PCR amplification. The latter was implemented doubly, firstly with initial 20 mM concentration of each of the primers in all sample tests, and secondly with higher initial concentration, 50 mM, of the primers to increase the

Table 1. Primer pairs used in RT-PCR amplification for detection of bovine *Rotavirus* A in clinical samples in this investigation.

	Nucleotide sequences of the applied RT-PCR primer pairs	3' Self complementarity	Tm	Product sizes (bps)	Origins
61F+62R	(5'à3') VP6 gene				
Forward	GGCTTTTAAACGAAGTCTTC	7	52.84	1356	Chinsangaram *et al*. (1995)
Reverse	GGTCACATCCTCTCACTACG	2	57.15		
Con2+Con3	(5'à3') (VP4 gene)				
Forward	ATTTCGGACCATTTATAACC	2	50.85	877	Gouvea *et al*. (1994)
Reverse	TGGCTTCGCCATTTTATAGACA	2	58.12		
Con2+OSU	(5'à3') VP4 gene				
Forward	ATTTCGGACCATTTATAACC	2	50.85	502	Gouvea *et al*. (1994)
Reverse	CTTTATCGGTGGAGAATACGTCAC	4	59.26		
63F+64R	(5'à3') VP6 gene				
Forward	TTGCGTCGGCAAGTACAGAT	2	60.04	453	Pham (2023a)
Reverse	TTCAGGTCGCTGGATTCGAC	2	60.11		
65F+66R	(5'à3') VP6 gene				
Forward	GATGCTAGAGACAAAATTGTCGAAGG	2	60.46	688	Pham (2023a*)
Reverse	CAGCTGAATTAATCACTCTTGGAAAAC	0	59.46		

*Outcomes of "Bovine/Cow" conservativeness and Primer Blast check

sensitivity of the reactions. The RT-PCR were then electrophoresed with either 6× GelRed loading buffer tricolor (ABT/Biotium Inc.,) or Safe Dye (P-Sdye-0250, Phusa Genomics) staining, screening under a UV source and photographed behind a UV shield (Pham, 2023a).

Determination of RVA specific antigens and antibodies

Detection of RVA antigens in fecal samples was performed by two ways. The first one was an immuno-chromatographic assay (ICA) with *Rotavirus* Rapid Test Cassette (Feces) kits, following the instructions of the provider (CiTest Laboratories Inc.). The second technique was the assay of Shifting of standardized direct hemagglutination inhibition (SSDHI) (Pham, 2023b). Related to this HA-based method, after the capacity of RotaTeq vaccine (Merck Sharp & Dohme Corp.) to agglutinating RBCs of chicken has been ascertained, HA reaction for determining the vaccine virus titers was performed, and then based on that success, with the participation of 4 HA unit titer suspensions of the viral vaccine, HI antibody titration was implemented with serum samples, on one hand, showed infected bovine individuals and, on the other, helped supply biomaterials for performing reactions of SSDHI in the next step for determining RV specific antigens in fecal samples. Also, with ICA, every RV antigen-positive diarrheic animal was planned to be checked daily during the course of diarrhea and through a week after the diarrhea finished.

Detection and titration of RVA specific antibodies was performed with hemagglutination inhibition (HI) reaction in 96-well microtiter plates for titrating RVA specific antibodies in bovine serum samples using 25 µL of 1% chicken red blood cell (RBC) suspensions and 25 µL of the vaccine solutions with 4 HA unit titer (2 $\log_2$ HA titer) in each plate well. The HI positive sera with high titers were collected, diluted and titrated to have antibody solutions of standardized 4 $\log_2$ HI units (16 HIU) and utilized as standard antibody solutions for SSDHI (Pham, 2023b) detecting relevant RV in the fecal samples as mentioned above.

Data analysis

Chi-square analysis with the aid of MS Excel software was applied for ratio comparison. Two ratios would be significantly different if chi-square test $P < 0.05$. And, the 95% confidence interval of a ratio is calculated as 95% CI = p ± 1,96×((p(1 - p)/N)^0,5), in which p is the rate of prevalence and N is the total number of samples (Snedecor and Cochran, 1980). A reference ratio could be considered to belong to the range of a studied population ratio if the value of the former falls within the 95% confidence interval of the latter.

Results

RT-PCR amplification of RVA VP gene fragments

With the pairs of primers, as shown in Table 2, applied in RT-PCR for amplification of the tested fecal samples, it was revealed that amplification of the target gene fragments with the three pairs of primers Con2+Con3, Con2+OSU, and 61F+62R showed all negative (Fig. 1-A, Fig. 1-C, and 1-C), i.e., with them all 197 samples were found negative. Even, higher concentrations, 50 pmol instead of 20 pmol, of the primer pairs of 61F+62R, the outcomes (Fig. 1-D) did not improve the sensitivity of the reactions but magnified "trash" products smaller than 400 bps, which might be mistakenly perceived as positive RT-PCR products in cases of lower molecular weights of targeted nucleotide fragments. However, with the both 63F+64R and 65F+66R primer pairs one diarrheic calf was coincidentally found infected with RVA (Fig. 1-E, lanes "79", and 1-F). It showed also that real positive cases have no "trash" bands, which can be a criterion for reading PCR outcomes. In addition, RT-PCR with 63F+64R, exemplarily shown here, demonstrated that RotaTeq vaccine was RT-PCR positive (Fig. 1-E, lane "IC") confirming the specificity of the gene fragment amplification product. So, there was only one calf amongst 49 diarrheic calves positive or amongst 197 bovines tested, i.e., 2.04% of diarrheic calves or 0.51% of cattle possessing the virus in their feces (Table 2).

Detection RVA-specific antigens in feces

The results of the application of CiTEST *Rotavirus* antigen ICA kits for detection of RVA in all the collected fecal samples showed that most of them were negative (Fig. 2) and only one calf, that was also observed as diarrheic, had the virus in its scour (Fig. 3-A). The outcomes of this antigen detection assay coincided absolutely with that of RT-PCR primed by the pairs of oligonucleotides 63F+64R and 65F+66R (Table 2). That is, only one calf was positive with the both techniques of ICA and RT-PCR, indicating again that the prevalence of RV infections reared in the area was 0.51% amongst susceptible cattle (including calves and gestating cows) and 2.04% amongst diarrheic calves at the moment of investigation.

Next, SSDHI, as shown in Fig. 4-C., demonstrated that the only positive calf with the two tests of RT-PCR and ICA was also positive with the technique of SSDHI (Table 2). All other fecal samples were also negative with this assay.

Finally, daily checking fecal samples of the only gene- and antigen-positive diarrheic calf showed the virus' presence in the neonate's scours during diarrhea (Fig. 3-A to 3-C) and one day after the disease

Table 2. Comparison of SSDHI test outcomes in detection of *Rotavirus* genes with RT-PCRs using adopted primer pairs (61F+62R, Con2+Con3, Con2+OSU, 63F+64R, 65F+66R) and *Rotavirus* antigens with ICA in bovine fecal samples.

		SSDHI		Prevalence (%)	Prevalence 95% CI (%)
		Positive	Negative		
61F+62R RT-PCR	Positive	0	0	0	
	Negative	1	196		
Con2+Con3 RT-PCR	Positive	0	0	0	
	Negative	1	196		
Con2+OSU RT-PCR	Positive	0	0	0	
	Negative	1	196		
63F+64R RT-PCR	Positive	1	0	0.51	-0.48 - 1,50
	Negative	0	196		
65F+66R RT-PCR	Positive	1	0	0.51	-0.48 - 1,50
	Negative	0	196		
ICA	Positive	1	0	0.51	-0.48 - 1,50
	Negative	0	196		
SSDHI	Positive	1	0	0.51	-0.48 - 1,50
	Negative	0	196		

stopped (Fig. 3-D). Further, ICA tests of the fecal samples collected showed that most of diarrheic cases in bovine reared here were not caused by RV.

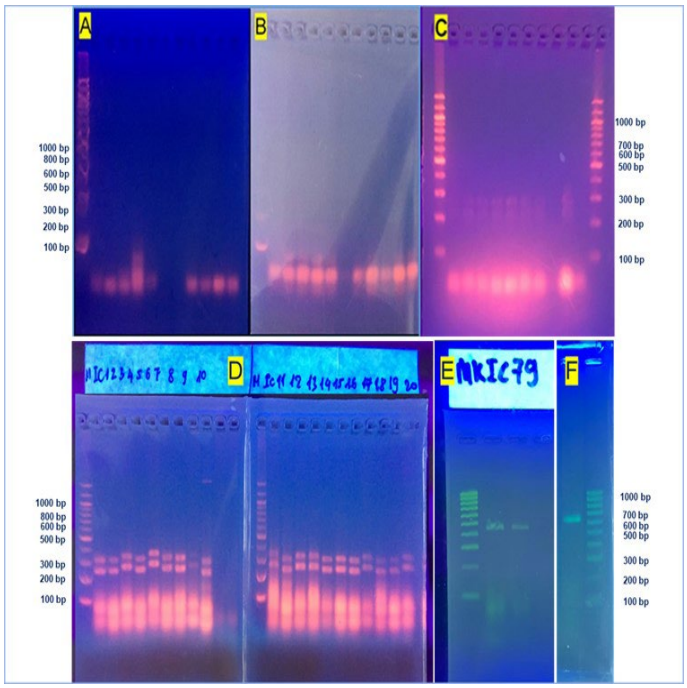

Fig. 1. Representative pictures showing outcomes of RT-PCRs for RV detection in calf and cow diarrheic and non-diarrheic feces. A, RVA VP6 gene RT-PCR outcomes amplified by primer pair 61F+62R with no expected bands of 1356 bp size products; B, RVA VP4 gene outcomes amplified by primer pair Con2+Con3 with no expected bands of 877 bp size products, and C, by primer pair Con2+OSU with no expected bands of 502 bp size products; almost all negative outcomes are with nonspecific "trash products" mainly smaller than 100 bps and fainter lower-than-400 bp bands (seen clearer in C); D, RVA VP6 gene RT-PCR products amplified by primer pair 61F+62R as in A but with higher concentrations of the primers (50 pmol instead of 20 pmol) with clearer nearly-to-400-bp non-specific trash bands; E, RT-PCR products by primer pair 63F+64R with the expected bands of 453 bp size parallel of a positive case (79) with a the band of RT-PCR product of RotaTeq vaccine virus as inner control (IC); and F, products by primer pair 65F+66R with the expected molecular weight 688 bp bands visualized. The letter M in gels stands for "molecular weight".

Determination of serum antibody conversion of bovine samples

As HA reactions (Fig. 4-A) for virus presence/titers were proven feasible with RotaTeq vaccine virus emulsions (Merck Sharp & Dohme Corp.), HI reactions for detection of antibodies specific to RotaTeq vaccine virus hemagglutinins were implemented (Fig. 4-B). Amongst 95 serum samples in the two stages of sampling (Table 4) there were 7 cases positive, indicating about 7.37% of the bovine population infected. This ratio has 95% CI range from 2.11% to 12.62%, statistically higher than the prevalence determined by both antigen detection (ICA, SSDHI) and gene amplification (RT-PCR) methods (P = 0.0008).

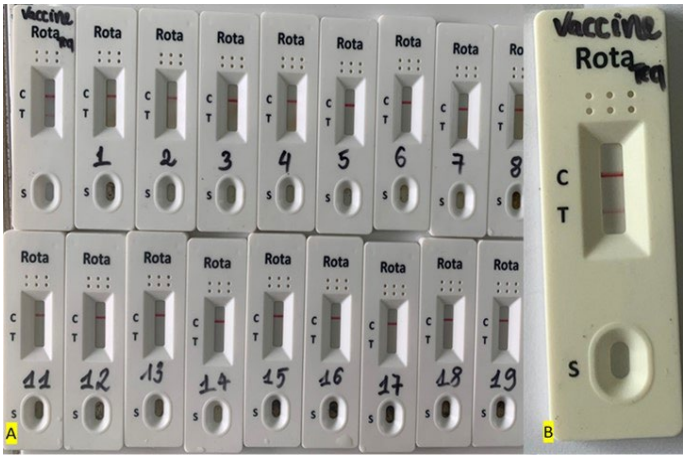

Fig. 2. Exemplar outcomes of ICA tests for the presence of RVA antigens. All cases in this series of tests were negative (A), while the inner control RotaTeq vaccine (A-upper left picture, and B) demonstrated positivity.

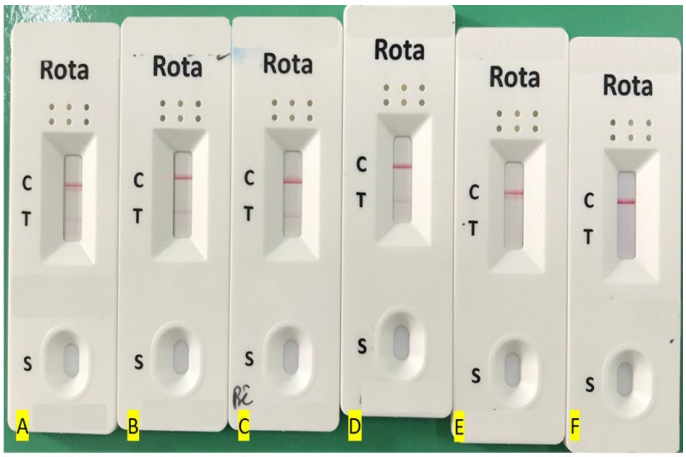

Fig. 3. Detection of RVA antigens in feces of a diarrheic calf with Immuno-chromatograhic assay (ICA). The positive appearance of virus antigens maintained during the course of the disease (A to C). The virus antigens were still present one day after the illness stopped (D) and disappeared since then (E, F), showing the virus' role as a causative agent of the disease in bovine.

Discussion

As RV strains causing infections in animals can either transmit directly to humans (Nakagomi *et al*., 1992) or supply one or more RNA segments for genome re-assortment, or genetic exchange, in human strains making infections productive (Cook *et al*., 2004; Dóró *et al*., 2015; Phan *et al*., 2016), investigation of virus prevalence in animals, hence, should be

Table 3. Prevalence of *Rotavirus* infection in groups of diarrheic and normal calves and gestating cows as determined by the combination of the outcomes of the RT-PCR, ICA, and SSDHI tests

Sample category	Number of tested samples	Number positive samples	Positive percentage (%)	95% confidence interval (%)
Diarrheic calves	49	1	2.04	-1.92 – +6.00
Normal calves	49	0	0	0
Diarrheic pregnant cows	50	0	0	0
Normal pregnant cows	49	0	0	0
Total	197	1	0.51	-0.58 – +1.50

Table 4. Serological prevalence of *Rotavirus* infection determined by HI tests in adult bovine reared in the studied area.

Sampling stages	Tested samples (heads)	Positive cases (heads)	Positive percentage (%)	95% confidence interval (%)
1 (2022)	50	3	6	-0.58 – +12.58
2 (2023)	45	4	8.89	+0.57 – +17.20
Total	95	7	7.37	+2.11 – +12.62

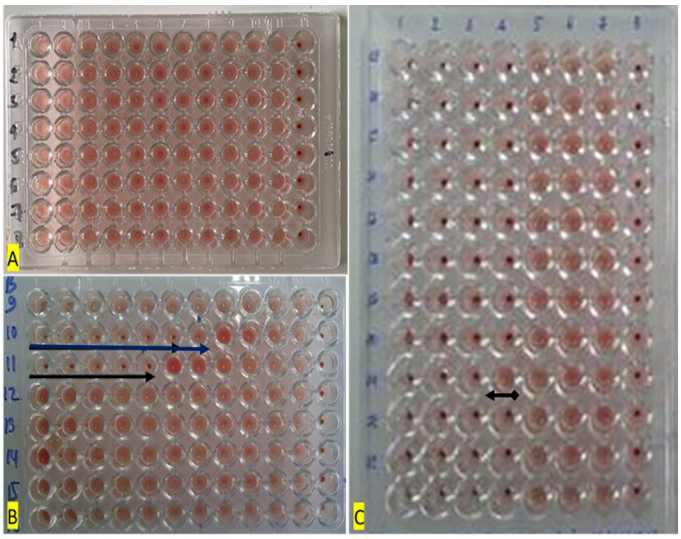

Fig. 4. Reactions of HA, HI and SSDHI. Expressions of HA for determining the titers of RotaTeq virus suspension showing high concentration of the virus in the vaccine (A), antibody-determining HI tests showing here two positive cases (B, shown by arrows), and SSDHI tests showing one sample positive (C, leftwards arrow). Note: all the rows of wells on each microtitration plate of this system of HA-HI-SSDHI have a cell sedimentation control in the rightmost well for determining proper initiation moment of reading the reactions.

useful for prevention of the infections in humans. This genus was investigated in many countries as the most common cause affecting almost one third of children hospitalized with severe diarrhea (Hallowell *et al*., 2022). In calves, as reviewed recently by Pham (2023a), it was determined present in many areas of the world in the past with different prevalence rates mostly higher than 40%. Based on the available database, it could be imagined that RV infections spread widely, and so finding the infectious agent in the nature could be easily succeeded. However, the rates of RV genetic and antigenic prevalence amongst cattle were found in this study only as 0.51% (95% CI ranges from -0.58 to +1.50%) and that amongst the diarrheic calf population was about 2.04% (95% CI ranges from -1.92 to +6.00) (Table 2). Meanwhile, the rate of bovines with antibody conversion obtained with the aid of HI (Table 4), i.e., the rate of bovines infected once in the past time long enough for virus antigen-induced formation of natural specific immunity since no vaccines were applied in the area, was 7.37%. Though with some increment to the RT-PCR and antigen detection tests' outcomes, the rate too low in comparison with those reported previously. With a 95% CI from +2.11% to +12.62%, it did not cover any of the values obtained by the reviewed researchers. Indeed, sampling procedures in most of those reports indicated that their tests were rather laboratory verifications of on-going diarrhea outbreaks than epidemiological investigations of common animal populations, as the clinical samples were mostly collected from apparently diseased animals. Likely, with RT-PCR a researcher group (Zaitoun *et al*., 2022) found that all of 16 already known serologically positive calves (100%) and 13 of them (81.25%) were positive with respectively RVA VP7 and VP4 gene RT-PCR amplification.

Especially, all the RT-PCR tests in this work with the first three primer pairs (Con2+Con3, Con2+OSU, and 61F+62R), particularly, resulted in all-negative outcomes: neither diarrheic calves nor pregnant cows were proven to possess the targeted virus' genes in their scours. The same phenomena were observed also in all the non-diarrheic animals. The discrepancies of the outcomes of the tests in this study with the previous researchers' obtainments, evoked suspicion that the outcomes of this study might be not natural, i.e., it could be so all-negative because the quality of the bio-materials applied in this study were possibly subjected to some dismays appeared during, e.g., the transportation of them from the providers to the local university-based laboratory. This hence suggested to verify the prevalence rates using different methods of detection of RV properties or components (antibody-induction, surface spike activities…) in general, as well as utilizing different primer pairs in RT-PCR methods, for minimizing possible errors of omission because of possible mismatching originated from the virus' genotypic diversity, which did eventually lead to the need of designing comprehensiveness-oriented primer pairs, such as 65F+66R pair (Pham, 2023a). Anyway, the applications of the antigen detection kit of ICA and the technique of SSDHI also resulted in such a low rate of prevalence of the infection (Table 2). The only positive calf determined by the comprehensively primed RT-PCR was also the only one that possessed the virus antigens in its scours as detected by both ICA and SSDHI techniques, which certainly targeted different molecules. Of the two antigen detection tests, SSDHI detection should be restricted to ascertaining the presence of only hemagglutinin-possessing strains.

Regarding to the use of HI and SSDHI for RV infection investigation, as indicated in a review of Patton (2012), RotaTeq vaccine was created by recombination of gene fragments of five VP6 type I2 RVA strains of human origin each possessing either one of G1, G2, G3, G4 or G6 gene types of VP7 protein integrated with a gene fragment encoding VP4 protein originated from bovine RVA strains with P[5] and P[8] genetic types. Besides, information from the RotaTeq vaccine producer (Merck Sharp & Dohme Corp. Leaflet V260-VNM-2018-016980) indicated that the orally recommended vaccine consists of G1, G2, G3, G4, and P1A[8] type strains (without G6 and P[5] gene type ones). It was established that "VP4 could determine the virulence, P-type, and HA property of *Rotavirus*" (Kalica *et al*., 1983), "HA by a human *Rotavirus* isolate could serve as evidence for transmission of animal *Rotavirus*es to humans" (Nakagomi *et al*., 1992), and "several human *Rotavirus*es (Wa, KUN, MO) could agglutinate only with fixed one-day-old chicken erythrocytes" (Fukudome *et al*., 1989). So, HA reaction of RotaTeq vaccine virus on fresh chicken RBCs imple-

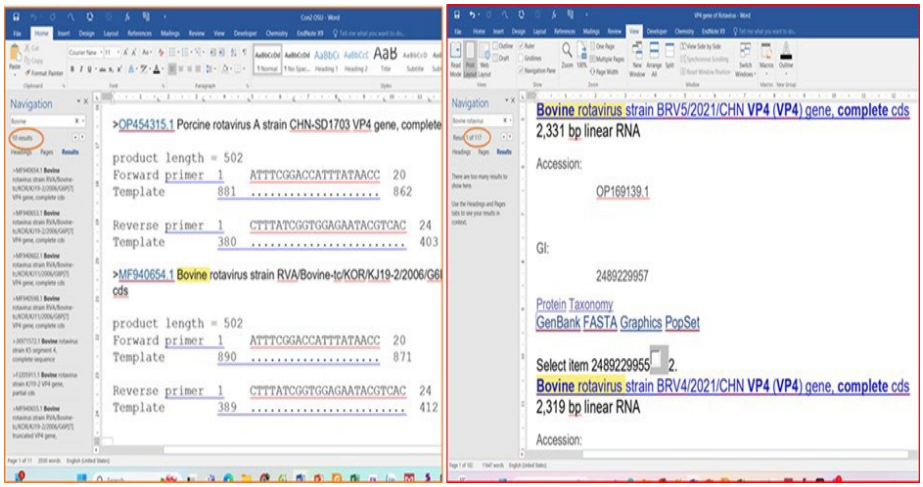

Fig. 5. The theoretical capacity of the primer pair of Con2+OSU in RT-PCR amplification of the relevant VP4 gene in bovines. Only 17 amongst 117 GenBank-databased bovine strains (14.53%) could be detected by RT-PCR amplification of the gene with the pair of primers.

mented here (Fig. 4-A) was caused by direct actions of hemagglutinins encoded by the re-assorted VP4 gene of bovine origin. Consequently, the outcomes of the HI and SSDHI assays in this investigation (Fig. 4-B, C) showed the rates of cattle infected with bovine RVA strains that possess hemagglutinins P1A[8], i.e., strains determined serologically as P1A and genetically as P[8] (Santos and Hoshino, 2005). Thus, application of the system HA-HI-SSDHI in investigation of RV is feasible. Of course, however, cases of infections with RV strains bearing VP4 spikes other than P1A[8] hemagglutinins could not be detected by HI and SSDHI tests based on the vaccine RotaTeq virus' HA property. So we should apply additional methods for comprehensive investigation on prevalence of the pathogen.

On the opposite, the ICA could be more comprehensive. It is depending upon the number of antigenic determinants serving as the targets of the antibodies applied in the device in the form of colorful nanoparticle-conjugated antibodies, which are placed next to the sample loading region and movable with loaded sample liquid alongside with other components by capillary action, and the targets of antibodies pre-coated on the test line region for trapping the specific antigens. In general, for the sake of the sensitivity and economy of ICA, the antibodies should be polyclonal antisera. RV-detecting ICA devices, however, if designed with polyclonal antibodies from whole RV particle vaccine-inoculated animals, could detect all RV strains possessing either one of the antigenic VPs, a priori particularly VP4 and VP7 on the virion surfaces. Among them, VP4 molecules have their roots between VP6 molecules of the virion's intermediate layer from the inside and protrude through the VP7 lattice shell (Sun *et al*., 2021) forming the viral spikes contributing to the viral attachment and penetration (Dormitzer *et al*., 2002). However, for ICA designed particularly to detect only the type A of the viral genus consisting of a vast number of types, from A to I, the applied antibodies must be specific only to type A VP6, which were produced by induction of animals with pure RVA-specific VP6 antigenic preparations free from any other antigenic proteins. As in the RV genus the VP gene fragments of the viral genome are discrete and so are able to re-assort independently, the ICA could detect all type A strains of RV regardless of hemagglutinins' presence, or, in other words, it can detect both hemagglutinating and non-hemagglutinating strains. The detected strains, so, could be other than those detected by SSDHI, which can detect hemagglutinins belonging to the type of the applied RotaTeq vaccine strain. This should partially resemble the research outcomes of Pisanelli *et al*. (2005) that showed that amongst 21 RVA ICA-positive isolates (16.7%) of 125 diarrheic fecal samples there 15 (71.4%) were VP4 P[5] and 6 (28.6%) were VP4 P[1] sharing the ICA-determined prevalence rate. The outcomes obtained with the three techniques of RVA antigen-specific ICA, RVA VP6-specific (63F+64R and 65F+66R-primed) RT-PCR, and VP4 P[8]-specific SSDHI in this study, however, coincided, implying the diversification of the virus is rather hindered. Next, the serological research with the P[8]-specific HI technique analyzing sera collected from another group of cattle in the same area population, though with statistically significant difference (P = 0.0008), showed only a slightly higher rate of prevalence (7.37%). So, the low prevalence rates of the viral infection could be the reality of RVA infections in bovine reared in the area. The 95% CI range of the prevalence rate in the second stage of serum sampling (from 0.57 to 17.20%) did cover the outcomes of several previous researchers such as 16.7% in Japan reported by Fukai *et al*. (1998) or 16.8% in Italy by Pisanelli *et al*. (2005). Also, the daily check of the virus in fecal samples during diarrhea and through a week after the stop of the illness using ICA showed that right one day after the diarrhea stopped the virus disappeared (Fig. 3). This fact, together with the low rate of prevalence of the viral infections, could mean both that the virus is primarily causative for the calf's diarrhea and that the presence of RV infection is real but with low contagiousness. Especially, applying RT-PCR with the primer pairs of 65F+66R, which is theoretically capable of amplifying a much broader range of RVA strains than 63F+64R (Pham, 2023a), also helped to confirm the above-mentioned outcomes of RV prevalence in locally reared healthy and diarrheic calves and cows at the moment of sampling.

Regarding to RT-PCR amplification, as showed in Table 1, the main possible factors causing negativity in the RVA detection outcomes, as failures of RT-PCR amplification, might be the effects of the combination of large gaps in melting temperatures (Tm) of either one oligonucleotide of each primer pairs with the applied primer-annealing temperatures (62°C) and the high values of their 3'-self complementarity. However, an analysis on the target coverages of primer pairs (Pham, 2023a) in comparison with the successes of the previous researchers in using the relevant primers showed that the comprehensiveness of the primers is more important for RT-PCR successes. Checking the capability of primer pair 63F+64R, for example, with the aid of Primer Blast (Ye *et al*., 2012), which supplied also lists of relevant sequences that could serve as potential target templates for PCR amplification, and searching nucleotide sequence list with the keywords "bovine/cow *Rotavirus*" on USA NCBI Nucleotide search engine (https://www.ncbi.nlm.nih.gov/nucleotide/) revealed that the number of bovine/cow RVA strains listed as strains having the analogous sequences amplifiable by the primer pair consists only 32% (16 amongst 49) bovine RVA strains possessing VP6 gene complete sequences available in GenBank (Pham 2023a). Similarly, with the pair of 61R+62F we could find only 55% (27 amongst 49) bovine-specified strains. This means that the number of the detectable strains is too smaller than the number of the to-be-detected strains. Furthermore, checking the capacity of the pairs of Con2+OSU and Con2+Con3 we found also the same phenomena of low coverages. For the pair of Con2+OSU that primarily used for RT-PCR detection of RV in diarrheic feces of children by amplifying a specific fragment of RV VP4 gene (Gouvea *et al*., 1994), checking for "bovine VP4 complete" in https://www.ncbi.nlm.nih.gov/tools/primer-blast/primertool... for possible target PCR templates and https://www.ncbi.nlm.nih.gov/nuccore/?term=bovine & VP4 & complete and then searching with the aid of MS Word Navigation for the number of bovine *Rotavirus* VP4 complete sequences could formulate a ratio of 10/117 (Fig. 5) theoretically implying that only about 8.55% bovine RV strains with GenBank-databased completely sequenced VP4 genes could be detected by RT-PCR with the primer pair. Similarly, with the pair of Con2+Con3 only 4 bovine- and 2 cow-related strains (0.05%) could be theoretically detected. Hence, with either of the primer pairs, there could be many bovine RVA strains omitted by RT-PCR. Probably, the strains that have been detected in this study by RT-PCR with either one of primer pairs 63F+64R and 65F+66R were those belonging to the series of omitted strains while tested with the mentioned primer pairs for VP4 gene detection. So, in responding to the inherent diversity of the virus, application of multiple RT-PCRs with comprehensive primer pairs targeting different gene fragments in combination with the other tests, including ICA, SSDHI, and HI, for detection of RV specific antigens and antibodies is advisable for epidemiological investigations. And, as shown in Fig. 1-A, B, C, and D, for low molecular weight target fragment RT-PCR cases trash products should be considered while reading lanes in electrophoresed gels.

Conclusion

At the moment of sample collection for this research the prevalence of RV infections was as low as 0.51% amongst calves and pregnant cows or as 2.04% amongst diarrheic calves as determined by RT-PCR, ICA, and SSDHI tests, though some RT-PCR primer pairs failed to amplify the relevant targeted genes of the pathogen's genome in fecal samples (showing 0%) possibly because of differences between the nucleotide sequences of primers and those of virus strains possibly present in samples. HI reactions with the aid of RotaTeq vaccine showed significantly (P = 0.0008) higher prevalence of RV in locally reared cattle in comparison with the RT-PCR and antigen-detecting assays. These rates (7.37% and 2.04%) of prevalence of RV, however, were lower than those reported so far by previous investigators in different regions of the world. Technically, the experiments showed that in response to the diversity of VP6 and VP4

genes' sequences of RV strains, RT-PCR primers for epidemiological investigation should be designed to detect as larger range of strain coverages as possible. In addition, HA, HI, and SSDHI using RotaTeq vaccine as an antigen source was feasible for RV investigation, of which SSDHI could succeed in detecting the virus strains in bovine feces, though only P[8] strains as determined by the hemagglutinin types of the vaccine virus used as standard antigens.

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Conflict of interest

There are no conflicts of interest to declare.

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